

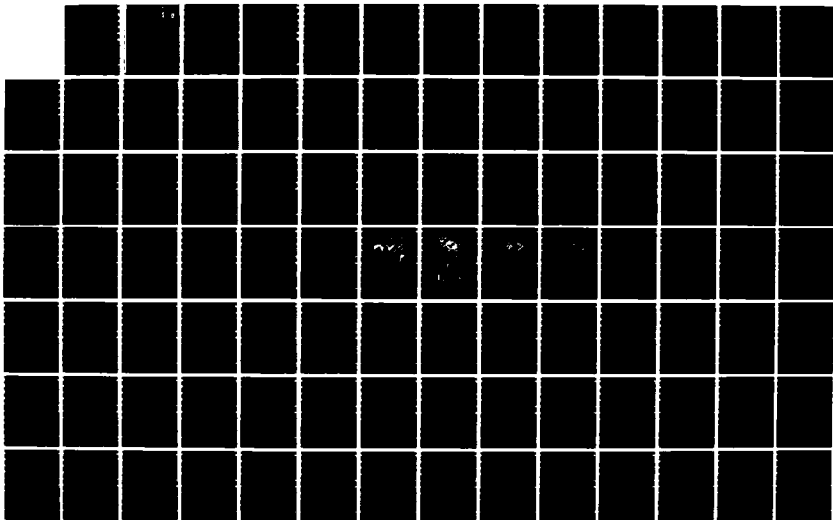
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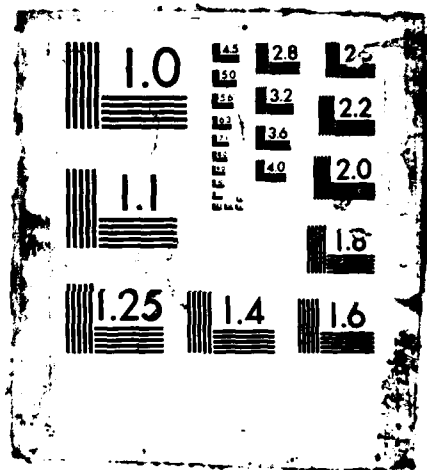
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FINAL PROJECT REPORT

HORMONAL-PHEROMONAL INTERRELATIONSHIPS

IN TICKS AND PARASITIC MITES

By

Daniel E. Sonenshine, Ph.D., Principal Investigator
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Submitted by the
Old Dominion University Research Foundation
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December, 1986



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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) → This final report summarizes the results of 6 years of investigations on the role of natural arthropod hormones on mating and reproduction in ticks and other Acari. Studies were directed to the role of steroid hormones, especially ecdysone and 20-hydroxyecdysone (20-OH ecdysone) and juvenile hormones, on these fundamental biological processes. Stimulation (excitation) of sex pheromone activity by 20-OH ecdysone, but not by juvenile hormones, was demonstrated, both in vivo and in vitro. Both females and males (the latter do not normally produce pheromone) respond to deliberate excitation by increased 2,6-dichloro-		

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phenol/tick sex pheromone) production. The histology and ultrastructure of the pheromone glands were described, and morphologic changes correlated with gland development during the ecdysial period, post-ecdysial period, maturation, feeding and repletion were noted. Studies on the biosynthesis of ecdysteroids demonstrated synthesis from cholesterol and implicated the synganglion-lateral segmental organ plexus as a major site for this activity. More recent studies using *in vitro* organ culture methods demonstrated a major role for the tick fat-body, which may serve as a site of synthesis, or release of stored hormone. Tick fat-body is a diffuse tissue, present in chords around the tracheae and on the surfaces of internal organs. The metabolism of ecdysteroids in ticks was described also. Following synthesis from cholesterol, ecdysone is metabolized to 20-OH ecdysone and, subsequently to a spectrum of non-polar ecdysteroid conjugates. The latter are produced by esterification with fatty acids. Saponification, or digestion with esterases, results in release of the free hormone, as well as other, more polar compounds that react in the radioimmunoassay (RIA). One of these compounds was tentatively identified as 20,26-dihydroxyecdysone by infrared and Nuclear Magnetic Resonance spectrometry. No evidence of 20-OH ecdysoneic acid was found. Although a variety of free ecdysteroids may exist in these ticks (20,26-di OH ecdysone, 20-OH ecdysone, ecdysone and, perhaps, others), the most abundant steroid was 20-OH ecdysone.

> The metabolism of exogenous juvenile hormone (JH) was also investigated by incubating authentic ^3H JH-3 with tick hemolymph. Chromatography of the products revealed 3 labelled metabolites, characteristic of the acid, diol and acid/diol. These findings suggest the presence of JH specific enzymes as well as general esterases capable of hydrolyzing this hormone. The variety of tick hemolymph proteins, including esterases, is described.

The potential use of ecdysteroid analogues to disrupt development and sex pheromone activity was investigated. Previous tests with the analogue BSEA-28 indicated substantial reduction in the ecdysial period in *H. dromedarii* nymphs and some disruption of feeding, as expressed by reduced body weight. Treatment with the analogue BSEA-1 (22,25 dideoxyecdysone) led to increased nymphal mortality following feeding, but only slight changes in the ecdysial period. When administered *in vivo* via the controlled release implant technique, both analogues, BSEA-28 and 22,25-DDE, led to significantly increased 2,6-dichlorophenol (2,6-DCP) production in females and excitation of 2,6-DCP production in males. Further evidence of the ability of ecdysteroids to excite 2,6-DCP production was obtained with treatments of pheromone glands maintained *in vitro*. Treatment with 22,25-DDE led to the highest levels of 2,6-DCP production ever observed in these systems.

New evidence of JH-like gonadotropic hormone was found in mites and ticks. Studies done by Dr. Oliver and his colleagues demonstrated partial loss of reproductive capacity in *Dermanyssus gallinae* treated with Precocene-2, and partial restoration of reproductive capacity by application of JH-III to the treated mites. In *Ornithodoros parkeri*, inoculation of JH stimulated vitellogenesis, but not oviposition. Preliminary evidence also implicated catecholamines in the initiation of vitellogenesis. Hemocoelic injections of male reproductive tissues into fed virgin females stimulated egg maturation and oviposition. Significant but less pronounced stimulation was also induced by inoculation of male salivary gland homogenates, but not by synganglial homogenates. Vaginal insertion of these extracts also induced oviposition. Other studies concerned the weight relationships between nymphal stages and subsequent stages and the processes of spermatogenesis and spermiogenesis. The number of chromosomes, autosomes vs. sex chromosomes, and the genetics of parthenogenesis is reviewed in several species. — could pg 115 Keywords: >

This project has led to the production of 50 scientific articles that have been published, or are in press, including 2 chapters in forthcoming books. In addition, 9 major symposium papers were presented at national and international meetings, as well as several short submitted papers presented before these societies. More publications and presentations are planned.

TABLE OF CONTENTS

	<u>Page</u>
ABSTRACT.....	ii
I. INTRODUCTION.....	1
II. PRODUCTIVITY OF THE HORMONE-PHEROMONE PROJECT.....	14
III. SUMMARY OF STUDIES ON THE ROLE OF ECDYSTEROIDS AND JUVENILE HORMONE(S) IN THE REGULATION OF SEX PHEROMONE ACTIVITY IN TICKS.....	21
IV. METABOLISM OF ECDYSTEROIDS IN TICKS.....	25
V. EVIDENCE OF ECDYSTEROID PRODUCTION BY TICK FAT BODY TISSUES MAINTAINED IN VITRO.....	31
VI. EFFECTS OF ECDYSTEROID ANALOGUES ON DEVELOPMENT AND SEX PHEROMONE ACTIVITY IN TICKS.....	49
VII. JUVENILE HORMONE(S) IN TICKS: EVIDENCE FOR OCCURRENCE AND POSSIBLE ROLE IN SEX PHEROMONE ACTIVITY.....	60
VIII. HORMONAL REGULATION OF REPRODUCTION IN TICKS AND PARASITIC MITES.....	80
IX. SUMMARY.....	86
X. LITERATURE CITED.....	89
DISTRIBUTION LIST.....	94

LIST OF TABLES

<u>Table</u>		<u>Page</u>
1	<u>In vitro</u> production of immunoreactive materials by organs or tissues maintained in culture as determined by radioimmunoassay.....	47
2	<u>In vitro</u> production of immunoreactive substances by organs or tissues maintained in culture as determined by radioimmunoassay (RIA)*,** Numbers represent picograms ecdysone equivalent/100 ul of incubation medium (Exp. 2).***, +.....	48
3	Effect of presence of the hormone analogue 22,25-dideoxyecdysone in rabbit hosts of <u>H. dromedarii</u> on survival and molting.....	56
4	2,6-dichlorophenol content of unfed <u>Hyalomma dromedarii</u>	

TABLE OF CONTENTS - Continued

LIST OF TABLES - Concluded

<u>Table</u>	<u>Page</u>
females from immatures to which ecdysteroids had been administered by various methods.....	57
5 2,6-dichlorophenol content of partially fed female <u>Hyalomma dromedarii</u> sex pheromone glands maintained <u>in vitro</u> following stimulation.....	58
6 <u>In vitro</u> stimulation of sex attractant pheromone activity, 2,6-dichlorophenol, in excised foveal glands of the camel ticks, <u>Hyalomma dromedarii</u>	59
7 Metabolism of juvenile hormone by hemolymph from part fed <u>Dermacentor variabilis</u> females.....	75
8 Metabolism of juvenile hormone by hemolymph from Tobacco hornworm <u>Manduca sexta</u> larval hemolymph.....	76
9. Metabolism of ^3H JH by Tobacco hornworm (<u>Manduca sexta</u>) hemolymph with fractions separated by TLC.....	77
10. Metabolism of ^3H JH-3 by part-fed virgin female <u>Dermacentor</u> various hemolymph with fractions separated by TLC.....	78
11. Effects of Treatment of <u>H. dromedarii</u> nymphs with JH-3 in DMSO:acetone (1:9) administered by the contact method on survival and the duration of ecdysis.....	79

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
1, 2. Light photomicrographs illustrating fibroblast-like cells in a primary culture initiated with mixed tissues associated with the fat body and epidermis from a virgin feeding female <u>Dermacentor variabilis</u> . Cells stained with methylene blue-Azure II. 120 X.....	37
3. Scanning electron micrograph illustrating the chords of fat body (fb) cells adjacent to the tracheae (T) near the tracheal trunks and spiracle of a partially fed virgin female <u>D. variabilis</u> . Such chords of cells were observed adjacent to tracheae attached to excised organs and surrounding the membranes of internal organs. 505 X.....	39
4-6. Transmission electron micrographs illustrating tick cells grown in cell cultures. 4. Cell from a culture initiated from excised tick organs as described in Fig. 1. Lipid	

TABLE OF CONTENTS - Continued

LIST OF FIGURES - Concluded

<u>Figure</u>	<u>Page</u>
droplets (ld) and rough endoplasmic reticulum are prominent features of these cells. 11,714 X. 5. Cell from primary culture of <i>D. variabilis</i> embryonic tissues, with numerous lipid droplets. 8,571 X. 6. Cell from culture of the continuous cell line, RML-19. 11,076 X.....	40
7-8. Transmission electron micrographs illustrating sections through the fat-body tissues adjacent to the tracheal trunks. 7. View of a group of fat-body cells adjacent to a section of a tracheae; T = tracheal lumen, cut = cuticle of tracheae, bl = basal lamina, rer = rough endoplasmic reticulum. 8,182 X. 8. Lobe of the fat body illustrating cytoplasmic features, especially abundant rough endoplasmic reticulum, vacuolation, channels or canaliculi (arrows), and lipid droplets; ld = lipid droplets, Nu = nucleus, v = vacuolations. 5,165 X.....	41
9. Fat-body cells at higher magnification demonstrating abundant accumulations of free ribosomes (fr) or polysomes; m = mitochondria. 20,571 X.....	42

I. INTRODUCTION

This final report summarizes the results of six years of investigations on the hormones and pheromones in ticks and parasitic mites. Hormones control various developmental processes as well as maturation of sperm and eggs. They also control production of sex pheromone. There are three major groups of hormones active in arthropods and they include the neurosecretory compounds, ecdysteroids and the juvenile hormone group. Based on our work and the publications of others, it seems that these three classes of hormones are also active in the Acari. The investigations at Old Dominion University concentrated primarily on tick pheromones and the role of steroid hormones, especially ecdysone and 20-hydroxyecdysone (20-OH ecdysone) and its relation to pheromone production. Studies were also conducted on the juvenile hormones. Investigations at Georgia Southern emphasized the mapping of the synganglion and its neurosecretory cells in a representative tick and mite species, spermatogenesis, oogenesis and oviposition and molting. Chromosomes, sex determining mechanisms and effects of gamma radiation and anti-juvenile hormone compounds on development and reproduction were also studied. In addition, pheromones in the poultry mite (Dermanyssus gallinae) and background studies dealing with reproductive strategies and sex determination were also conducted.

Stimulation (excitation) of sex pheromone activity by 20-OH ecdysone, but not by juvenile hormones, was demonstrated, both in vivo and in vitro. Both females and males (the latter do not normally produce pheromone) respond to deliberate excitation by increased 2, 6-dichlorophenol (tick sex pheromone) production. The histology and ultrastructure of the pheromone

glands were described, and morphologic changes correlated with gland development during the ecdysial period, post-ecdysial period, maturation, feeding and repletion were noted. Studies on the biosynthesis of ecdysteroids demonstrated synthesis from cholesterol and implicated the synganglion-lateral segmental organ plexus as a major site for this activity. More recent studies using in vitro organ culture methods demonstrated a major role for the tick fat-body, which may serve as the site of synthesis or release of stored hormone. Tick fat-body is a diffuse tissue, present in chords around the tracheae and on the surface of internal organs. The metabolism of ecdysteroids in ticks was described also. Following synthesis from cholesterol, ecdysone is metabolized to 20-OH ecdysone and, subsequently, to a spectrum of non-polar ecdysteroid conjugates. The latter are produced by esterification with fatty acids. Saponification or digestion with esterases results in release of the free hormone, as well as other, more polar compounds that react in the radioimmunoassay (RIA). One of these compounds was tentatively identified as 20, 26-dihydroxyecdysone by infrared, and Nuclear Magnetic Resonance spectrometrics. No evidence of 20-OH ecdysonic acid was found. Although a variety of free ecdysteroids may exist in these ticks (20, 26-di OH ecdysone, 20-OH ecdysone, ecdysone and, perhaps, others), the most abundant steroid was 20-OH ecdysone.

The metabolism of exogenous juvenile hormone (JH) was also investigated by incubating authentic ^3H JH-3 with tick hemolymph. Chromatography of the products revealed three labelled metabolites, characteristic of the acid, diol and acid, diol. These findings suggest the presence of JH specific enzymes as well as general esterases capable of hydrolyzing this hormone. The variety of tick hemolymph proteins, including esterases, is described.

The potential use of ecdysteroid analogues to disrupt development and

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When administered in vivo via the controlled release implant technique, both analogues, BSEA-28 and 22,25-DDE, led to significantly increased 2,6-dichlorophenol (2,6-DCP) production in females and excitation of 2,6-DCP production in males. Further evidence of the ability of ecdysteroids to excite 2,6-DCP production was obtained with treatments of pheromone glands maintained in vitro. Treatment with 22,25-DDE led to the highest levels of 2,6-DCP production ever observed in these systems.

In order to understand the interrelationships among neurosecretory cells, ecdysteroids and juvenile hormones, it was first necessary to learn about the location and activity cycles of the neurosecretory cells. Therefore, histochemical investigations were conducted. Single esophageal and paired cheliceral, palpal, pedal (I-IV), and opisthosomal nerves enter the synganglion and form specific neuropilar ganglia. The ganglia are integrated by a complex series of commissures and connectives. Eighteen paraldehyde-fuchsin-positive neurosecretory regions, which vary greatly in size and amount of granular neurosecretory material, are each associated (one or more) with neuropilar ganglia. Presumably transport of neurosecretory materials to target tissues occurs through axonal pathways, perineurial-neural lamella associations, and the neurohemal retrocerebral organ complex.

The retrocerebral organ complex (ROC) in penultimate nymphal and adult

female Ornithodoros parkeri is composed of a single dorsal, and paired ventral, lobes. The dorsal lobe lies anteriorly adjacent to the postero-dorsal extremity of the oesophagus, in a position immediately ventral to the proventriculus, and is innervated by a pair of compound nerves. Single branches of these nerves connect the dorsal lobe to the paired ventral lobes which lie at the posterolateral margins of the oesophagus. Histologically, the ROC in O. parkeri females appears similar to those reported in other species.

Histological techniques and paraldehyde-fuchsin (PAF) staining were used to study the synganglion and to locate neurosecretory regions and neurosecretion within the synganglion of the chicken mite, Dermanyssus gallinae. The synganglion, which is formed internally by neuropillar ganglia, gives rise to a single esophageal and paired cheliceral, palpal, pedal (I-IV), and opisthosomal nerves. The neuropilar ganglia are interconnected by commissures and connectives within the synganglion. Twelve PAF-positive neurosecretory regions are present in unfed protonymphs, unfed deutonymphs, virgin males and females, and mated males. There are 11 PAF-positive neurosecretory regions in larvae, 24-72 hours post-fed deutonymphs and mated females. Neurosecretory regions in these developmental stadia are described in relation to their positions adjacent to individual neuropilar ganglia.

Our work and the results of others have shown that ecdysteroids exert a regulatory control over molting in ticks. No one, however, has previously reported induction of apolysis and cuticle deposition as a result of injection of 20-OH ecdysone in ticks. We found that five concentrations (0.001, 0.01, . 0.1, 1.0, and 10.0 $\mu\text{g}/\mu\text{l}$ olive oil, injected into the hemocoels of

virgin female O. parkeri 48 hours postfeeding caused apolysis and new epicuticle formation. The percentage of ticks forming new cuticle increases up to the 0.1 $\mu\text{g}/\mu\text{l}$ oil concentration and decreases slightly at the 1.0- and 10.0 μg concentrations. Mortality in some ticks was caused by doses of 0.1, 1.0, and 10.0 μg concentrations. These results not only prove a regulatory role for this hormone, but also suggest that recently fed female O. parkeri ticks might provide a useful bioassay of ecdysteroids. We also developed another useful experimental technique, i.e. membrane feeding of O. parkeri and the chicken mite, Dermanyssus gallinae. Membrane feeding by haematophagous arthropods provides a means of studying their role in disease transmission, their nutritional requirements, as well as various physiological, developmental, and behavioral events. We found that all developmental stages and adults of O. parkeri feed readily through membranes on defibrinated bovine, swine, and chicken blood. Ticks feeding on bovine and swine blood molt or oviposit, but most ticks that feed on the chicken blood die. Refrigerated or frozen bovine blood remains acceptable and nutritionally adequate for 16+ days. Natural membranes serve better than synthetic ones for tick feeding, and preliminary experiments indicate that 34-35 C is the optimum temperature for in vitro feeding of O. parkeri. Ingested 20-OH ecdysone has an effect on both nymphs and adult females. Most third stage nymphs which remain nymphs after molting (i.e., did not produce adults), molted a second time if they had received a sufficiently high concentration of ecdysone. Adult females molted or attempted to molt after ingesting 20-OH ecdysone at a high enough concentration. However, many did not respond to this treatment by molting, and most of those attempting to molt had been only partially successful. Interestingly, we have discovered another regulatory role of 20-OH ecdysone. Evidence to be presented later suggests that

it triggers spermatogenesis in ticks.

Cytokinetic analyses were carried out on the testis of the American dog tick (Dermacentor variabilis) nymph during the developmental period which follows attachment to the host and feeding. The spermatogonial cell division cycle in vivo was determined: Tc, 22.8 hr; S, 6.7 hr; G1, 8.2 hr; G2, 4.0 hr; P, 3.5 hr; M, 0.4 hr. Histological techniques were used to determine the timing of spermatocyte differentiation and accumulation. This accumulation is markedly biphasic, occurring as two distinct waves during the developmental period. Models of germarial zone division-induction were derived, and theoretical population curves generated by these models were compared to the empirical data.

Development of a previously-unreported secretory tissue in close association with the nymphal testis was described, and some correlations between germarial division activity and endocrine events known from related species compared.

A count of spermatocysts in the recently-ecdysed adult male Dermacentor variabilis indicates that spermatogonial mitosis continues at a high rate for the first few days of adult life and then declines to a low level. An earlier proposed model of spermatocyte accumulation was corrected for this activity and the theoretical curve generated by the model was compared to the empirical data.

[³H]-TdR labelling demonstrates a continuous low level of DNA synthesis during the adult pre-feeding period, although the cell population remains stable. The rate of DNA synthesis suggests that the spermatocyte pool is completely turned over about every two months. Degeneration of cysts of primary spermatocytes at the end of the 'small growth' period in the posterior extremity of the testis tubule is described and suggested to be an

essential component in the maintenance of this stable spermatocyte pool in the light of a continued input through spermatogonial mitosis.

A five-fold increase in spermatogonial division activity follows feeding. In addition, a marked decrease in duration of the first meiotic prophase also occurs.

In addition to supplying nutritional needs for spermatid development, the blood meal likely serves to activate other stimuli necessary for spermatogenesis to occur, i.e., hormones. As already noted, approximately 400 germ cells are active in DNA synthesis in each testis of unfed male D. variabilis, and upon feeding for 24 hours, approximately 1,000 cells are labeled; by the third day of feeding, 2,000 cells are labeled, and as many as 2,500 labeled cells can be found subsequently during feeding. We suspected that an ecdysteroid might play a role in early phases of spermatogenesis and thus injected three concentrations of 20-OH ecdysone into unfed male D. variabilis. We also injected synganglion extract made from feeding males. Results demonstrate an approximate doubling of DNA synthetic cells by extracts of the equivalent of 0.1 synganglia from feeding males. 20-OH ecdysone had an extremely potent effect on germ cell DNA synthesis; injections of 0.2 µg/tick resulted in numbers of cells synthesizing DNA not significantly different from the numbers of such cells in 3-5 day feeding adults and four times that of control ticks. Lower doses of 0.05 µg/tick produced a half-maximal stimulation of approximately 1,000 cells and a higher dose of 0.5 µg/tick produced only a marginal response as measured by this assay. Injected saline and muscle extracts were without effect.

The foregoing experiment clearly demonstrated the competence of the germ cells in the adult testis to respond to 20-OH ecdysone. It remained to be shown that the adult actually produced such a hormone. A bioassay for

presence of the hormone was devised consisting of implanting tissue from nymphs to feeding adult males. We reasoned that nymphal cuticle and its hypodermis are known to respond to 20-OH ecdysone by undergoing ecdysis in the nymph and that this tissue should respond similarly if exposed to the putative 20-OH ecdysone in feeding males. Since males do not imbibe large quantities of blood, and thus internal body pressure does not stretch the cuticle much, we performed the following experiment. The scutal cuticle plus its hypodermis was removed from unfed nymphs and placed over a window cut in the scutum of unfed adult males after surface sterilization of both nymphs and adults. The edges of the wound were sealed with Tackiwax (Cenco, Montreal), and males were allowed four days to recover from surgery. The males were then allowed to attach to a rabbit for four days at which time they were forceably detached, their scutum removed under cold, osmotically buffered fixative (Karnovsky's fluid modified by exclusion of acrolein and addition of 0.2M sucrose), embedded in JB 4 Resin (Polysciences, Warrington, Pennsylvania), sectioned at 2 μ m and stained with toluidine blue or Hansen's trioxymatin. In all cases a new cuticle had been produced. However, cellular hyperplasia at the edges of the wound made it impossible to determine if the hypodermal cells producing the new cuticle were derived from the nymphal transplant or from the adult recipient. Therefore, a series of implants from adult to adult was then prepared and treated as before; no new cuticle could be discerned in any of the four preparations. Transplants of either nymphal or adult hypodermis and cuticle to adult males which were allowed to recover from surgery and then held unfed for four days, showed no new cuticle production.

The experiments involving 20-OH ecdysone injections demonstrate that adult testis is responsive to 20-OH ecdysone in concentrations which compare

favorably with those which stimulate similar responses in testis of larval and nymphal insects, and that the response is not directly dependent on nutritional adequacy. The experiments involving transplants of nymphal cuticle to males and then allowing them to feed show that not only is adult testis responsive to 20-OH ecdysone, but that feeding males produce it. Such a presence of ecdysteroids in a non-molting male might be unexpected, but it is known from males of this species according to others. Unfortunately, other experiments (Oliver unpublished) involving injection of various concentrations of 20-OH ecdysone into unfed males did not result in significant increase in number of spermatogonia and spermatocytes at metaphase. Nevertheless, experiments of Dumser and Oliver (unpublished) did show that at least one aspect of spermatogenesis, increase in the number of germ cells synthesizing DNA, is responsive to hormones. Also, the increase of labeled cells noted upon injection of crude synganglion extract suggests a prothoracicotropic effect of brain hormone similar to that found in insects. However, a direct effect of the latter extract on the testis or on other organ systems, such as fat-body, cannot be ruled out with such in vivo experiments.

There is an extensive and remarkable morphogenesis of tick spermatids during their period of maturation (capacitation of some authors). Prior to transfer to the female, spermatids remain in the seminal vesicle and show no motility or additional morphological change regardless of time spent there. Upon transfer to the female, maturation results in rupture of an operculum at the anterior end of the spermatid followed by eversion of the anterior end and subsequent elongation to approximately twice its original length. Maturation of tick spermatids occurring after transfer to the female is induced by secretions of the male genital accessory gland which are added to

the spermatids within the spermatophore during transfer to the female. Apparently, although the secretions of the accessory gland are adequate to induce the entire developmental changes of the spermatids into spermatozoa, their specific action is to induce only the opening of the operculum. The accessory gland secretion responsible for the remarkable maturation of spermatids of Ornithodoros moubata (Argasidae) and Dermacentor variabilis (Ixodidae) is a polypeptide with a molecular weight of approximately 12,500. The polypeptides from the two species are similar in most respects, however, there is a lack of cross-specificity, and the one from O. moubata will not activate spermatids from D. variabilis and vice versa. Thus despite their similarity, obvious differences exist in the two compounds.

Both feeding and mating are necessary for successful oviposition in most species of ticks (excluding parthenogenetic and autogenous species). Reproductive strategies differ between the Argasidae and Ixodidae, and a complex interplay among various stimuli involved in feeding, mating, and oviposition occurs in different species. Induction of oogenesis and oviposition are two distinct phenomena and are usually controlled by different stimuli.

We found evidence of a JH-like gonadotrophic hormone in the chicken mite, D. gallinae. Mites treated with the antiallatropic precocene-2 lose their reproductive capacity, but when subsequently treated with JH-3, part of the reproductive capacity is restored. In the soft tick, O. parkeri, females treated with precocene-2 results in undeveloped ovaries, but subsequent treatment with JH reestablishes vitellogenesis and fully formed ovaries and eggs result. Cancellation of the anti-juvenile hormone effects of precocene-2 and the reestablishment of oogenesis (and oviposition in one specimen) by a naturally occurring insect JH argues strongly for a

physiological role of JH in acarine reproduction. Preliminary evidence also implicates catecholamines (L-DOPA, dopamine, epinephrine, norepinephrine) and prostaglandin E2 initiate early stages of vitellogenesis. Hemocoelic injections of male reproductive tissues into fed, virgin females also stimulate egg maturation and oviposition. Significant, but less pronounced stimulation was also induced by inoculation of male salivary gland homogenates. Vaginal insertion of these extracts also induced oviposition.

Probably catecholamines, perhaps prostaglandins and almost certainly a juvenile hormone-like gonadotrophic hormone, regulate egg maturation and oviposition. Vitellogenesis and its control may be very similar to that found in insects, whereby the fat-body produces most of the vitellogenins under control of a JH or a JH-like compound. Precisely where this JH-like compound is produced, how it is regulated, and its interrelationships with neurosecretory materials and ecdysones remain to be discovered. There seems to be a complicated interplay among nervous, endocrine, and reproductive systems prior to and during egg maturation and oviposition.

The effects of different temperatures on the expression of autogenic development in unfed/mated Ornithodoros parkeri Cooley females were examined. High temperature (29 C) induced autogeny in lower, middle and higher weight classes (11-20, 21-30, 31-40 mg, respectively) with all females in the highest weight class and most in the middle weight class ovipositing within 51 days post-mate. At a lower temperature (21 C) autogenic development was not expressed by higher and middle weight females, whereas lower weight females exhibited similar degrees of autogeny at both temperatures.

There is an interesting relationship between weights of nymphal stages and subsequent development of O. parkeri. Unfed 3rd-instar nymphal

Ornithodoros parkeri (UN3s) were arranged into seven weight classes, fed, and reared to adults. Fed 3rd-instar nymphs (N3s) produced 22.0% males, 3.8% females, and 57.2% 4th-instar nymphs (N4s); 17.0% died or were lost. The resulting N4s were fed and subsequently produced 14.7% males, 56.4% females, and 9.5% 5th-instar nymphs (N5s); 19.3% died or were lost. The N5s were fed and yielded 5.0% males and 70.0% females; 25.0% died or were lost. After rearing all UN3s to the adult stage, the final sex ratio was 0.77 male:1 female (43.5% male, 56.5% female). UN3s weighing less than 2.6 mg produced males and N4s but no females after feeding. A few females were produced from N3s heavier than 2.6 mg. N4s resulting from all N3 weight classes developed into males, females, and N5s, and those originating from N3s in the lowest weight class (0.1-1.0 mg) produced more males than females. N4s originating from all other N3 weight classes produced more females than males. In a second experiment, only females ecdysed from fed N4s weighing 25 mg and over, while those weighing 5-9 mg produced only males and N5s. The N5s in both experiments produced females almost exclusively (34 females and 1 male).

Pheromones play a role in regulating aggregation in mites. Dermanyssus gallinae mites consistently aggregate and form clusters of mixed developmental stages; recently fed mites aggregate most quickly and form more definite clusters than unfed mites. Thigmokinesis and pheromones are involved in the clustering behavior. The aggregation pheromone(s) is volatile and can be recognized by mites without directly contacting it. D. gallinae is not attracted (or if so, only weakly) to several synthetic chemicals that are reported to function as pheromones in other acarine taxa. It is somewhat attracted to synthetic guanine.

Sex determining mechanisms and timing of gametogenesis were determined

in five species of Amblyomma, O. parkeri and D. gallinae. Once the normal developmental and spermatogenic pattern was established for these species, gamma radiation was applied to A. americanum and D. gallinae to determine what effects it would have on these parameters. No recognizable alterations or changes in timing of spermatogenesis was noted from ticks treated with 0.5, 1, 2, 3, 4, 8, and 16 krads, but severe breakdown and depletion of germinal cells was noted at 4, 8, and 16 krads. Also, testis of ticks treated with 2, 3, 4, 8, and 16 krads were smaller than those of ticks not treated. Sterility in males was noted at treatment of 2 krads or higher. In D. gallinae, 3 krads reduced the number of treated protonymphs attaining adulthood and 6 krad treatment prevented all mites from developing to the adult stage. Egg production was normal for mites treated with 0.50 krads, but significantly curtailed by doses of 0.75 krads and greater. Radiation doses used in this study did not appear to affect sex ratio.

II. PRODUCTIVITY OF THE HORMONE-PHEROMONE PROJECT

During the 6-year period of this project, we have published 50 articles concerning work done on this subject, including chapters in nationally recognized review journals, proceedings of International Congresses, chapters in books, and original articles in prestigious journals. In addition, the Principal Investigator and Co-Principal Investigator presented invited papers at major national and internal symposia during meetings of relevant societies, specifically, the Entomological Society of America, International Congress of Entomology (XVII), VIth International Congress of Acarology, American Society of Parasitologists, and the IInd International Congress of Chemical Ecology. The Principal Investigator and Co-Principal Investigator (Dr. Oliver) presented invited symposium papers at the joint plenary session of the American Society of Parasitology and The American Society of Tropical Medicine and Hygiene (800 members) in Denver, Colorado to 800 members, and the Principal Investigator presented an invited paper at the Pheromone Biochemistry symposium of the Entomological Society of America in Reno, Nevada, both in December 1986. Other presentations were given at local or regional society meetings.

A complete list of original articles, book chapters, proceedings and other publications follows. In addition, a selected list of invited symposium or plenary session presentations at major societal meetings is also given.

LIST OF PUBLICATIONS, MANUSCRIPTS, AND INVITED SYMPOSIUM
PRESENTATIONS ON WORK SUPPORTED BY N-00014-80-C-0546

- 1981 Sonenshine, D.E., P.J. Homsher, J.S. VandeBerg and D. Dawson. Fine structure of the foveal glands and foveae dorsales of the American dog tick, Dermacentor variabilis (Say). J. Parasitol.
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- Sonenshine, D.E., G.M. Khalil, P.J. Homsher, W.H. Dees, K.A. Carson, and V. Wang. IBID. II. Maturation and pheromone activity.

IBID 20: 424-439.

Gaber, S.H.A., G.M. Khalil, D.E. Sonenshine, and M.A. Moez. Precocene-2 effects on the camel tick, Hyalomma dromedarii. 1. Adult responses. IBID 20: 534-540.

Khalil, G.M., S.H.A. Gaber, D.E. Sonenshine, and S.M. Gad. IBID. 2. Larval and nymphal responses. IBID 20:

Silverstein, R.M., J.R. West, G.M. Khalil, and D.E. Sonenshine. Occurrence of 2,6-dichlorophenol in the hard ticks Hyalomma dromedarii and Hyalomma anatolicum excavatum with notes on its role in mating regulation in these species. J. Chem. Ecol. 9: 1543-1549.

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Pound, J.M., J.H. Oliver, Jr. Morphology of the retrocerebral organ complex in penultimate nymphal and adult female Ornithodoros parkeri (Cooley) (Acari: Argasidae). p. 295-303. IBID.

Campbell, J.D. and J.H. Oliver, Jr. Membrane feeding and developmental effects of ingested B-ecdysone on Ornithodoros parkeri (Acari: Argasidae). p. 393-399. IBID.

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- 1982 Sonenshine, D.E. Pheromones of the Acari and their potential use in biological control strategies. Invited Symposium presentation at the VI the Internatl. Congr. Acarol., Edinburgh, Scotland, Sept. 1982 (subsequently published by Ellis Horwood).
- 1983 Sonenshine, D.E., P.J. Homsher and K.A. Carson. Evidence of the role of tick chelicerae in perception of genital sex pheromones. Entomol. Soc. Amer. meetings, Detroit, MI, Nov., 1983.
- 1983 Oliver, J.H., Jr. and B.F. Stone. Spermatid production in unfed metastriata ticks. J. Parasitol. 69: 420-421.
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- 1984 Sonenshine, D.E. Two invited symposium presentations at the XVII International Congress of Entomology, Hamburg, FRG (West Germany), August, 1984: (1) "Courtship behavior and reproductive isolation of species, Section 8 (Developmental Biology). This was one of only two background papers and was allotted 40 minutes (others only 20 min). (2) "Tick Pheromones: An Overview" Section 15 (Recent Advances in Morphology, Physiology and Behavioral Biology of Ticks). This was a 20 minute paper.
- The latter paper has been published in the proceedings of the Congress.
- 1985 Oliver, J.H., Jr. and J.M. Pound. Cytogenetics of ticks (Acari: Ixodoidea). 16. Chromosomes and timing of spermatogenesis in Amblyomma inornatum. J. Parasitol. 71: 124-126.
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- 1985 Sonenshine, D.E. and D. Taylor. "Semiochemical mediated adaptations of the Acari for finding hosts and mates". International Society of Chemical Ecology, University of Wisconsin, Madison, Wisconsin, June 8-12, 1985. Published as a separate paper (see above).

Sonenshine, D.E. "Hormonal regulation of mating behavior in ticks" in the President's Symposium", "Highlights in Recent Research on Ticks and Tickborne Diseases" American Society of Parasitologists, Athens, Georgia, August, 1985.

Sonenshine, D.E., Beveridge, M., Taylor, D. and Upchurch, B. Metabolism of ecdysteroids in ticks. Submitted paper presented at the Entomological Society of America meetings in Miami, FL, December 1985 (Section B).

- 1986 Sonenshine, D.E. Sex pheromones: the chemical basis of mate finding strategies in ticks. Presentation before the joint opening plenary session of the American Society of Parasitologists and the American Society of Tropical Medicine and Hygiene, Harry Hoogstraal Symposium. This was a major presentation before 800 members of the two societies; this presentation was one of only 4 papers scheduled. Presented at the joint meetings of the two societies in Denver, Colorado, December, 1986.

Oliver, J.H. Tick evolution: Host preferences, chromosomes, development and reproductive strategies. Presentation before the joint plenary session of the American Society of Parasitologists and the American Society of Tropical Medicine and Hygiene. Same symposium as above, with the same opportunities to present our work before the 800 members assembled in this session.

Sonenshine, D.E. Neuroendocrine regulation of sex pheromone behavior in ticks. 30 minute presentation in the Symposium "Pheromone Biochemistry", (Section B), Entomological Society of America annual meetings, Reno, Nevada, December, 1986.

III. SUMMARY OF STUDIES ON ECDYSTEROID REGULATION OF SEX PHEROMONE ACTIVITY

Evidence of direct stimulation of secretion and biosynthesis of sex pheromone, 2,6-dichlorophenol (2,6-DCP), was described by Dees et al. (1984a), who observed increased female attraction of mate-seeking males, increased pheromone content, and even stimulation of 2,6-DCP secretion. Unfed H. dromedarii females, which normally do not secrete pheromone or attract males, exhibited a 3.6-fold increase in 2,6-DCP content and significant sex attractant activity after treatment with exogenous 20-E. They even observed stimulation of 2,6-DCP production in male ticks, a life stage which has vestigial pheromone glands, but which normally does not produce this chlorinated phenol. Subsequent studies (Dees et al., 1984b,c) described the changes in ecdysteroid content in the different life stages, but failed to reveal a direct correlation between 20-E concentration and sex pheromone production. Nevertheless, these findings represent the first known evidence of hormonal stimulation of sex pheromone activity in ticks. In a followup study, Sonenshine et al. (1985) found that monoamines, believed to be catecholamines, stimulated sex pheromone activity. Using histofluorescence, x-ray microanalysis and gas chromatographic techniques to compare monoamine and 2,6-DCP occurrence, these authors demonstrated decreased monoamine and pheromone occurrence in response to the catecholamine-depleting drug reserpine and the antagonist (of dopamine biosynthesis) alpha-methyl-tyrosine-methyl ester, but elevated responses when ticks were treated in vivo with dopamine. In a recent review, Sonenshine (1985) hypothesized a model in which 20-E acts on the neurons to stimulate increased production of dopa-decarboxylase at the axonal termini, in a manner similar to that suggested by Schlager et al. (1974) for mosquitoes. Of course, the potential role of

neuropeptides and peptide hormones generally is not excluded.

Independent evidence of ecdysteroid stimulation of sex pheromone activity was described by Blomquist and his colleagues in a series of papers dealing with the sex pheromone of the housefly, Musca domestica (Blomquist et al., 1984a,b; Adams et al., 1984a,b). Although a detailed, in-depth review of their work is not essential here, several key findings that relate to similar evidence of 20-E regulation of sex pheromone biosynthesis in ticks is worth noting. In houseflies, a series of hydrocarbons in the cuticular waxes provide the female sex pheromones that attract males. The active components include (Z)-9-tricosene (the major component), tricosene-10-one, and 9,10-epoxytricosene. In the presence of 20-E, synthesis of these compounds was found to proceed in the insect epidermis. Ovariectomy of the flies terminated synthesis and led to elongation of the carbon chain, thereby bypassing the ^{23}C molecule, (Z)-9-tricosene. Addition of 20-E to the ovariectomized flies restored the synthesis of this pheromone. Moreover, treatment of male flies with this hormone induced pheromone biosynthesis in this sex, which normally lacks the pheromone. Transplantation of ovaries or ovarian homogenates into male flies produced the same effect. Clearly, the hormone directs the synthesis of this pheromone, a classic expression of the steroid-hormone model described below. Moreover, the receptor-acceptor binding sites are so precise as to affect synthesis of only a single enzyme, the decarboxylase responsible for converting the methyl-branched alkane precursor to the ^{23}C side chain bearing molecule (Z)-9-tricosene. Thus, in the synthesis of the pheromone from oleic acid, the major substrate, chain-lengthening proceeds incrementally to ^{23}C , whereupon decarboxylation and branching generates (Z)-9-tricosene in the presence of 20-E. In the absence of 20-E, extended chain lengthening to

²⁷C or longer hydrocarbons occurs. The importance of these findings by Blomquist and his colleagues is that they demonstrate ecdysteroid stimulation of sex pheromone production in a different arthropod than the tick, and provide a detailed description of the biochemical events. Presumably, specific receptors exist in the epidermal cells, where the pheromone is synthesized, which bind 20-E, translocate to the nucleus, and lead to the production of specific RNA that codes for the specific decarboxylase needed for these syntheses. Thus, the evidence for ticks and insects converge, providing strong evidence that 20-E is the specific excitant responsible for sex pheromone biosynthesis.

These findings from ticks and muscoid flies support Kittredge and Takahashi's (1972) remarkably prescient theory of the adaptation of ecdysone/20-E for regulation of mating behavior in terrestrial arthropods. These authors suggest that the archetypal sex pheromone was 20-OH ecdysone, which was secreted into the surrounding water by aquatic arthropods preparing for their final molt. With the evolution of terrestrial arthropods, new types of pheromones more suited to life on land were needed. Continuing production of ecdysone and 20-E in adult animals provided the means for regulating the activities of the glands involved in mating and reproduction, i.e., the pheromone glands and the gonads, while the evolution of receptor proteins and the sensitivity of DNA-transcriptase systems to the hormones provided mechanisms of action. The evolution of the foveal glands in ticks greatly resembling the dermal glands so common throughout the epidermis of these animals, is readily appreciated when one considers that the epidermis is the predominant target organ for ecdysteroids throughout the life cycle (e.g., its role as "the" molting hormone). In turn, stimulation or blocking of this action by juvenile hormone, now acting as gonadotropic hormone,

would mediate the timing of this process. Thus, a system of stimulant and suppressor molecules, operating with appropriate feedback loops, provides the broad framework within which sex pheromone activity may proceed. In ticks, neurosecretory centers are also involved, and neurosecretions transferred by the foveal nerve axons appear at the axonal termini. These neurosecretions appear to serve primarily as stimulations for secretion, since 2,6-DCP is already present in the glands. Thus, the outlines of a regulatory system appear to exist in these animals, providing a model which may now be tested in vivo and in vitro. Study of sex pheromone biosynthesis, especially with in vitro culture methods, should be especially rewarding.

IV. METABOLISM OF ECDYSTEROIDS IN TICKS

In contrast with the insects, little was known about the metabolism of ecdysteroids in ticks when this project commenced in 1980. In insects, ecdysone is hydroxylated by ^{20}C hydroxylase to 20-hydroxyecdysone mostly in peripheral tissues such as the fat body. Ecdysone and 20-hydroxyecdysone may be hydroxylated further to 20,26-dihydroxyecdysone. Ecdysone and 20-hydroxyecdysone may be degraded to their 3-dehydro forms (Riddiford and Truman, 1978), as well as 3-epi and/or 26-hydroxy derivatives (Dinan et al., 1981), and/or 2-deoxyecdysone and 2-deoxy-20hydroxyecdysone (Isaac et al., 1983). In some insects, e.g., the desert locust, Schistocerca gregaria, inactivation is also accomplished by the formation of highly polar conjugates, primarily as sulfates of ecdysone, 20-hydroxyecdysone, or their metabolites (Koolman et al., 1973), or as phosphate esters (Isaac et al., 1982, 1983), or even as adenosene monophosphoric esters (Tsoupras et al., 1983). Other routes of inactivation involve acetylation at the 3-position as well as conjugation with phosphate (Isaac et al., 1984) and the formation of highly polar, non-hydrolyzable ecdysanoic acids (Lafont et al., 1983). In contrast to the sulfate or phosphate conjugates, which may provide a source of active hormone by enzymatic hydrolysis in a later life stage, conversion to the acid forms appears to be irreversible.

In the locust, Schistocerca gregaria, the major inactivation products are formed by esterification with inorganic phosphate in the ^{22}C position and passed to the eggs; ^{22}C phosphate esters of ecdysone, 20-OH ecdysone, 2-deoxyecdysone, and 2-deoxy-20-OH-ecdysone have been reported (Isaac et al., 1982, 1983). The high reactivity of the ^{22}C position appears to account for the particular form of these conjugates. These conjugates are readily hydrolyzed by esterases, liberating the original ecdysteroid moieties.

Thus, they can provide a convenient storage form for the active hormones for later use (e.g., in the late stage embryo) as well as a means of enhancing synthesis by removal of free steroid from the active synthetic tissues (Isaac et al., 1983). Further studies with related orthopterans revealed other phosphate conjugates, especially 22-adenosine monophosphate-2-deoxyecdysone, 3-acetyl-ecdysone-2-phosphate, 3-acetyl-20-OH-ecdysone-phosphate, 3- and 2-acetyl-ecdysone, and 3-epi-2-deoxyecdysone-2-phosphate (Isaac et al., 1983, 1984; Isaac and Rees, 1984). These latter compounds were considered as irreversible inactivation products of the parent molecules. Clearly, the 2^{C} , 3^{C} , and 22^{C} positions appear to be the major sites where conjugates may form. Further degradation yields ecdysanoic acid and 20-OH ecdysanoic acid (Isaac and Rees, 1984), as described more fully below.

Lafont and his colleagues have shown that one of the principal routes of inactivation of ecdysteroids is oxidation to the corresponding acids. Following synthesis of ecdysone and its hydroxylation to 20-OH ecdysone by ecdysone 20-monooxygenase, these compounds are oxidized further to 26-OH ecdysanoic and 20,26-di-OH-ecdysone. In those insects in which metabolites lacking a 25-hydroxyl group occur, e.g., Ponasterone A, the metabolite is converted to inokosterone by hydroxylation at the 26^{C} position and then to the corresponding acid, 25-deoxy-20-OH-ecdysanoic acid. Frequently, these metabolites, as well as the parent molecules, form relatively polar conjugates with glucuronic acid, glucosides, sulfates, and phosphates. In addition, Lafont and Koolman (1983) noted that conjugation with amino acids and even with glutathione also occurs in some insects, a reaction which transforms the ecdysteroids into very polar conjugates.

In locusts, 2^{C} phosphate conjugates of 2-deoxyecdysone, 2-deoxy-20-OH-ecdysone, and 20-OH-ecdysone represent the predominant form of these

ecdysteroids, although phosphate esters at $2C$ and $3C$ are also known. Moreover, ester formation with adenosine monophosphate at the $22C$ position and acetate at the $3C$ position are also common. Side chain cleavage also occurs (forming poststerone), however, this happens only rarely. Finally, fatty acyl esters formed with various ecdysones at the $22C$ position have been described in ticks (see below), leading to the formation of highly polar ecdysteroids.

LaFont et al. also describe the major enzyme systems responsible for these pathways. Ecdysone-20-hydroxylase, found in the fat body, midgut, and many other tissues, hydroxylates ecdysone, forming 20-OH ecdysone. Ecdysone oxidase leads to the various deoxy ecdysone species, while ecdysone epimerase generates the 3-epi-ecdysteroids, and 2-dehydroecdysone reductases result in the various 3-dehydroecdysones. Conjugates are formed by the corresponding transferase enzymes, i.e., glucosyl, sulfo, phosphotransferases, and so on. The enzymes responsible for catabolism to the various ecdysanoic acids or esterification with fatty acids are unknown. All of these enzyme systems are cytosolic enzymes, so that the sites of ecdysone activity and breakdown are intracellular.

The metabolism of ecdysteroids in ticks appears to be very different from that observed with insects. At present, only four studies on this subject in ticks have been reported, one by Wigglesworth et al. (1985) on the cattle tick, Boophilus microplus, and others by Bouvier et al. (1982), Connat et al. (1984), and Dienl et al. (1985) on the soft tick O. moubata. In the case of B. microplus, very little activity (approximately six percent) corresponding to polar conjugates or acidic forms of ecdysteroids was found. Rather, most of the 3H radioactivity observed following injection of 3H ecdysone was correlated with ecdysone, ecdysone 3-acetate, and three

major peaks of relatively apolar material which were not immunoreactive. These occurred in both the parent females and the newly-laid eggs (probably formed maternally). Presumably, the apolar fractions contain metabolites of the parent ecdysteroids. The susceptibility of the apolar compounds to hydrolysis by esterases and by alkaline saponification followed by the release of free ecdysteroids suggests that they are acyl fatty esters with ecdysteroid moieties. Moreover, the unhydrolyzed apolar compounds can be transformed into acetonide derivatives, which suggests that the ^{22}C and ^{3}C positions are not substituted. Consequently, the authors concluded that the apolar compounds are ^{22}C fatty acyl esters of ecdysteroids. Similar findings were made in O. moubata by Bouvier et al. (1982), Connat et al. (1984), and Diehl et al. (1985), who demonstrated that most (70 to 75 percent) of the ^3H ecdysone injected into the ticks was converted into 2 relatively apolar fractions, AP-1 and AP-2. Enzymatic hydrolysis (with esterase) liberated free ^3H ecdysone and ^3H 20-OH ecdysone. In subsequent studies, Diehl and his colleagues (1985) reported evidence of 4 major conjugates consisting of 20-OH ecdysone esterified at ^{22}C with palmitic, stearic, oleic, and linoleic acids. Although it is not clear which of these fatty acids are present in which proportions in the two different classes, AP-2, the least polar of the four classes, is incorporated preferentially into the vitellogenic ovary, passed to the embryos, and linked to the vitellogenic process in some unknown way. Clearly, these compounds may serve in some capacity other than merely as inactivation products. Diehl et al. (1985) postulated that this mechanism may occur in the midgut as a means of inactivating exogenous ecdysteroids present in the blood of herbivorous hosts, i.e., for coping with phytoecdysteroids.

In summary, both families of ticks possess enzymatic pathways for

inactivation ecdysteroids by esterifying them at the 2^2C position, most probably with fatty acids. The ecdysteroid moiety undergoes little change, although the existence of other metabolites is not precluded. In this respect, ticks appear to differ greatly from most insects which degrade the parent molecule into a spectrum of acidic, dehydro, and deoxy forms and further inactivate these metabolites, as well as the parent molecules, by conjugation. However, this mechanism is not unique to ticks, as similar apolar compounds have been found in Drosophila (reported by Diehl et al., 1985).

Our studies with H. dromedarii suggest a metabolic fate for ecdysteroids similar to that described in B. microplus and O. moubata. In H. dromedarii, as in the other ticks, the 2 largest ecdysteroid fractions were apolar, representing almost 75 percent of the entire extract, and saponification led to the release of 20-OH ecdysone. We have been unable, however, to detect evidence of high molecular weight fatty acids following alkaline hydrolysis nor have we detected any indication of 2^2C or 3^3C acetate ecdysteroids. Hydrolysis of the tick extract with Helix glucuronidase/sulfatase enzymic preparation had no apparent effect on the amounts of immunoreactive ecdysteroids detected, a finding which leads us to exclude ecdysteroid conjugation with polar moieties such as phosphate, sulfate, or glucuronates. Significant amounts (up to 12 percent) of compounds more polar than 20-OH ecdysone were found to contain ecdysteroid moieties when examined by proton NMR and infrared spectroscopy. Comparison of their retention times with known standards suggests that at least one of them may be 20,26-OH ecdysone. The identity of the apolar compounds is unknown, although the presence of 1^4C labelling in the same fractions as those showing 3^3H labelling certainly implicates fatty acids.

The physiological effects of the various ecdysteroids other than 20-OH ecdysone in ticks is unknown. It is not known whether these are merely inactivation products, storage products retained in an inactive form for later use, or some combination of the two. Moreover, it is not known to what extent these varied ecdysteroids may be sequestered preferentially in selected tissues or organs, e.g., the vitellogenic ovary, midgut, etc., where they may continue to exert an effect.

A comprehensive, detailed study of this subject was presented in a separate publication (Sonenshine et al. 1986, J. Med. Entomol. 23:630-650).

V. EVIDENCE OF ECDYSTEROID PRODUCTION BY TICK FAT BODY TISSUES MAINTAINED IN VITRO

Introduction

In insects, ecdysone is produced by the prothoracic gland during larval development. In some insect species, it is also produced by the ovary in the adult female. Ecdysone is metabolized in the fat-body and other target organs to 20-hydroxyecdysone (20-OH ecdysone), the active hormone regulating molting, vitellogenesis, and in some species, sex pheromone production (Hagedorn 1985).

No glands analogous to the insect prothoracic glands have been found in ticks, and the source of ecdysteroids in these important parasites remains unclear,. Structures in the tick synganglion believed to serve as neuro-hemal or putative endocrine organs were described by Saito (1960), Eichenberger, (1970), Binnington & Tatchell (1973), Chow & Wang (1974), Obenchain & Oliver (1975), Paniflova (1978), Roshdy et al. (1973), Binnington (1981), Pound (1981), Pound & Oliver (1982) and others. Evidence implicating tick synganglion as a possible source of molting hormone was described by Cox (1960). The endocrine character of the lateral segmental organs was described by Binnington (1981) and Marzouk et al. (1985). However, no evidence implicating these glandular structures as a source of ecdysteroids was described in any of these reports. Sonenshine et al. (1985) reported greater accumulations of radioimmunoassay (RIA) positive material, believed to be ecdysteroids, in extracts of tick synganglia and lateral organ nerve plexus tissues than in other internal organs. Another possible source of ecdysteroid synthesis in ticks is the fat-body. This

tissue is concentrated in chords of cells around the tracheae as well as diffusely distributed throughout the tick body (Obenchain & Oliver 1973). Ellis & Obenchain (1984) reported immunoreactive material in media used to incubate fat body and other tissues excised from nymphal Amblyomma vari-
egatum Fabricius. However, it was not clear whether the fat body cells, rather than other cells that proliferated in vitro, produced the immunoreactive material.

The present paper reports studies to determine whether a specific organ or tissue may be a source of ecdysteroids in ticks, producing (or releasing) these steroids in vitro, or whether fat body associated with these structures produces these compounds. We also considered the possibility that cells proliferating from these cultures are the source of the ecdysteroids.

Materials and Methods

Ticks. Dermacentor variabilis (Say) was colonized and reared as described previously (Sonenshine et al. 1977). Unfed adult females of uniform age (2-3 wks postmolting) and were held in an AMINCO-AIRE controlled-environment chamber at 27 ± 1 °C and $90 \pm 2\%$ RH before feeding. After ticks were allowed to feed on albino rabbits (Oryctolagus cuniculus) for 7 days, they were forcibly detached and collected for dissection.

Dissection and organ collection. Partially fed ticks were surface sterilized as described by Yunker & Meibos (1979). Hemolymph was collected from a severed leg at the coxal joint in a heat-sterilized Drummond microcapillary pipette (VWR Scientific, San Francisco, CA).

Desired tick organs or tissues were aseptically excised in a sterile hood. Filter sterilized Shen's solution (Oliver, 1972) was used to wash tissues free of midgut fragments or hemolymph. Samples of the midgut, salivary glands, fat body, hemolymph (100 ul) and complete ovaries and synganglia were removed, washed 2 X in sterile Shen's solution, rinsed in filter-sterilized Yunker-Meibos culture medium (Yunker & Meibos 1979), and transferred to sterile Falcon culture plates (Becton-Dickinson and Co., Oxnard, CA). Amounts of material in each organ or tissue sample used in experiments 1 and 2 and numbers of ticks used are described in Tables 1 and 2. In experiment 3, a total of 25 synganglia (including lateral plexus), 100 ul hemolymph, and tracheae/fat-body tissues from 10 ticks were used.

Maintenance of excised organs/tissues in vitro. The excised organs were incubated at 27 °C in the wells of the culture plate. In experiment 1 the organs were divided into duplicate wells, each with 0.2 ml of culture medium; in experiments 2 and 3 the organs of a particular type were incubated together in the same well with 0.5 ml of culture medium. One day after incubation began, 50-ul (exp. 1) or 100-ul (exp. 2 and 3) aliquots were removed from each well for RIA (see below); wells were replenished immediately with an equivalent amount of fresh medium. This procedure was repeated on alternate days for 7 days (exp. 1) or 13 days (exp. 2 and 3), as shown in the tables. Controls consisted of wells with culture medium only.

Histologic studies. Excised organs or tissues were removed at the end of the incubation period, fixed in 10% neutral buffered-formalin, dehydrated, embedded in paraffin, sectioned, and stained (hematoxylin and eosin) (Humason 1972). The stained sections were examined by light microscopy. Controls consisted of the same organs and tissues from other partially fed

individuals but fixed immediately without incubation.

Tissue culture. Primary cultures were initiated with organ/tissue samples from partially fed virgin females as described above. Experiment 3 was done with these primary cultures. In that experiment, the organ or tissue samples were kept in the wells to allow cells to detach from these sources and reattach to the culture well surfaces. These fibroblast-like cells proliferated extensively, forming a monolayer within 3-5 days. The original organs or tissues were then removed and the primary cultures incubated for an additional 14 days. For comparison, cultures of the D. variabilis continuous cell line (RML-19), donated by Dr. C.E. Yunker, and a primary culture from D. variabilis embryonic cells (EC) were also maintained and assayed. Aliquots of the culture medium, 100 μ l each, were removed for assay on alternate days as described above.

The primary cell cultures were viewed with a Nikon Type 108 Diaphot-TMD inverted microscope and photographed with a Nikon M-35FA 35-mm camera.

Radioimmunoassay (RIA). RIA was done as described by Dees et al. (1984a) and modified by Sonenshine et al. (1985b). The 50-or 100- μ l aliquots of medium were extracted (methanol), reconstituted in 10% methanol, and the fraction containing the ecdysteroid isolated by eluting the extract from Waters C-18 Sep Paks (Waters Assoc., Inc., Milford, MA) with 75% methanol. The methanolic eluate was dried, reconstituted in borate buffer, and assayed as described by Dees et al. (1984a).

Ultrastructure. Samples of excised organs were examined for evidence of attached fat-body cells by scanning electron microscopy (SEM). The excised organs were immersed immediately in cold 2.5% glutaraldehyde-2% formaldehyde buffered with 0.1 M cacodylate (4 °C, pH 7.4) for 2-3 h, washed 3 X in 0.1 M cacodylate buffer, and postfixed for 2 h in 1% OsO₄ in 0.1 M

cacodylate buffer. After 2 washes of 5 min each in buffer, the tissues were dehydrated with ethanol followed by acetone and critical point dried with liquid CO₂ in a Denton DCP critical point drying apparatus (Denton Vacuum Systems, Cherry Hill, NJ). The dehydrated tissues were mounted on aluminum stubs with TV-corona dope (GC Electronics, Rockford, IL) and coated with 100-200 Å of gold-palladium in a Polaron E-5200 sputter coater (Technics Co., Alexandria, VA). The specimens were viewed with a Cambridge Model S-100 scanning electron microscope.

For transmission electron microscopy (TEM) of the cultured cells, cells growing in suspension were pelleted by gentle centrifugation, fixed, osmicated, and dehydrated as described above. The cells were infiltrated overnight with 1:1 acetone:Polybed 812 and embedded in Polybed 812. For TEM of the fat-body tissues, samples of the tracheal trunks and tracheae adjacent to the spiracles were excised from partially fed virgin females, fixed in cold 2.5% glutaraldehyde-2% formaldehyde, processed as described, embedded in Polybed and processed as described by Sonenshine et al. (1983). The specimens were viewed and photographed using a Hitachi HU-11 B transmission microscope.

Results

In vitro production of ecdysteroids by tick tissues. The viability of the cultured organs and tissues was evidenced by observation of muscle contractions and heart pulsations at 3-5 days and the normal histologic morphology of the cells at the end of the experiments (after at least 7 days).

Table 1 summarizes the results of studies with D. variabilis organs or tissues maintained in culture for 7 days (exp. 1). These results suggest

the production (or release) of immunoreactive material, presumably ecdysteroids, by all 5 organs or tissue types; the results for the controls were all negative (not included in the table). In Table 1, the 1st column (obs.) under each organ or tissue type, shows the concentrations of immunoreactive material, expressed in picograms of ecdysone equivalents/100 ul of incubation medium. The 2nd column shows the reduction in the concentrations expected on subsequent observation days if no new synthesis and/or release had occurred. If no new release had occurred, repeated dilution of the original culture with fresh medium on each of the observation days would have led to the reductions shown in this column. Substantial variations occurred from one observation day to the next. With the exception of the tracheal trunk-/fat-body cluster, all of the organ cultures showed the greatest concentrations of ecdysteroid on day 7.

Table 2 summarizes the results of a similar experiment extended to 13 days (exp. 2). Foveal glands and surrounding epidermal tissues were also incubated. The results demonstrate continued production (or release) of ecdysteroids throughout the 13-day incubation period, although the amounts produced were generally less than in the preceding experiment.

No evidence of ecdysteroid production was observed in cultures originating from hemolymph after the plasma and unattached cells were removed (exp. 3).

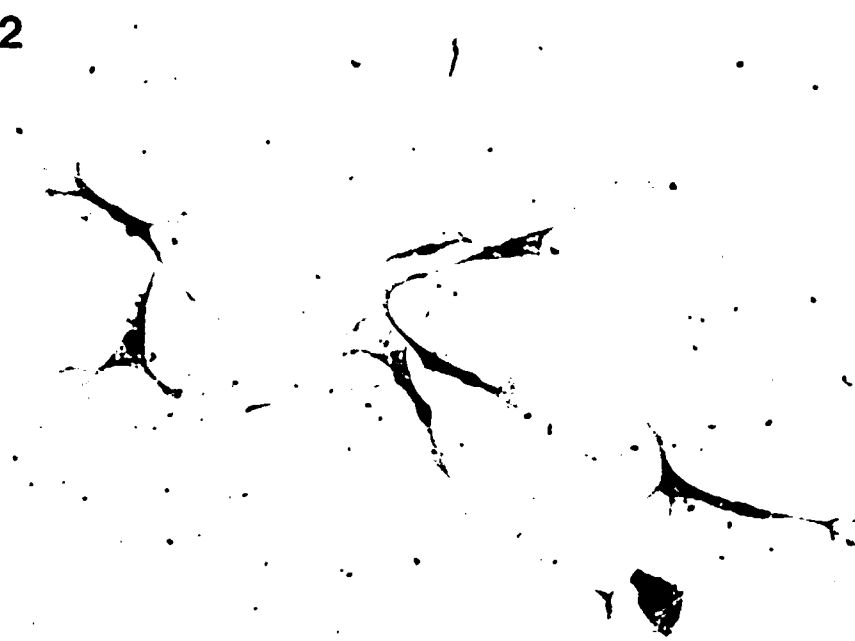
Primary cell cultures proliferated in all culture wells and in many cases reached confluency. Many cells adhering to the bottom of each well exhibited a fibroblast-like morphology (Fig. 1, 2), while those floating free in the medium appeared nearly spherical. However, in contrast with the results obtained in the previous experiments, no ecdysteroids were detected when the excised organs or tissues (including hemolymph) were removed

Figures 1, 2. Light photomicrographs illustrating fibroblast-like cells in a primary culture initiated with mixed tissues associated with the fat body and epidermis from a virgin feeding female Dermacentor variabilis. Cells stained with methylene blue-Azure II. 120 X.

1



2



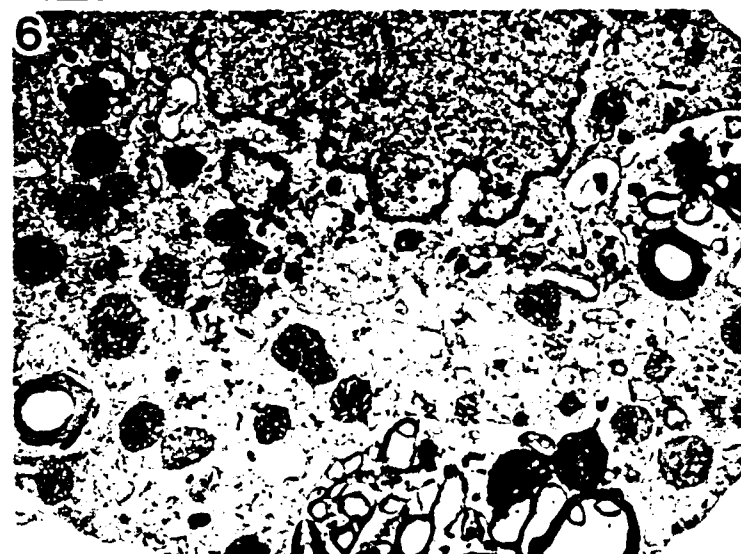
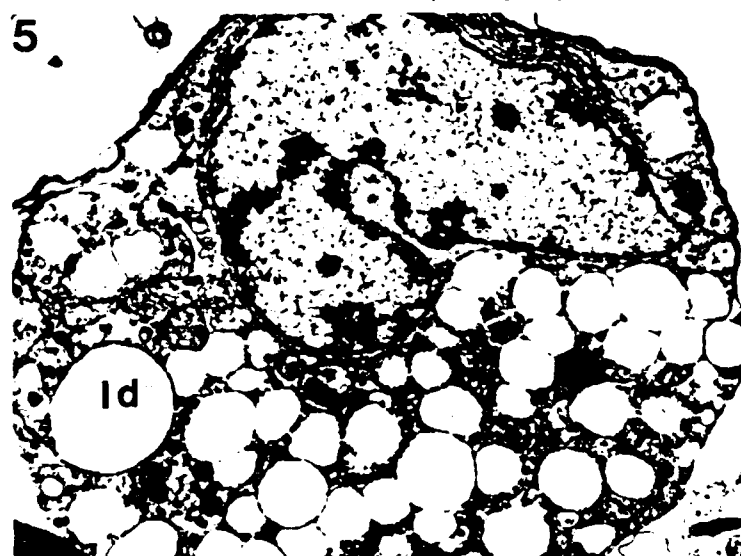
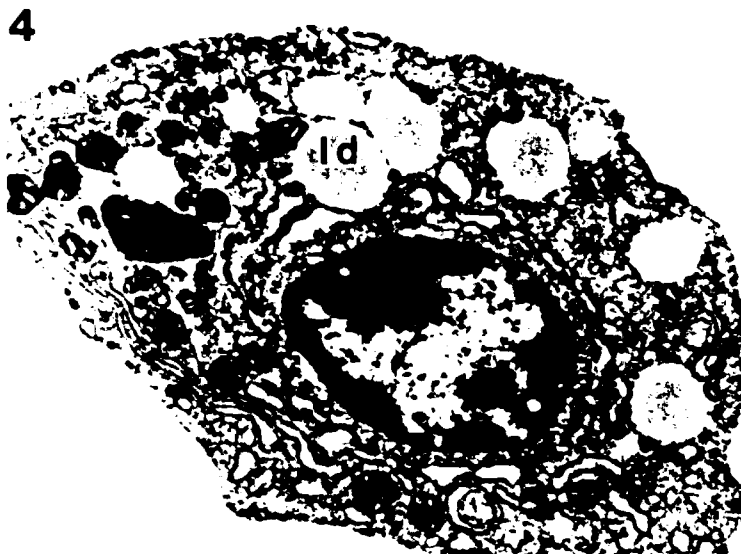
following primary cell multiplication (exp. 3). Similarly, no ecdysteroids were found in the media supporting cultures initiated with tick embryos or with the continuous tick cell line, RML-19.

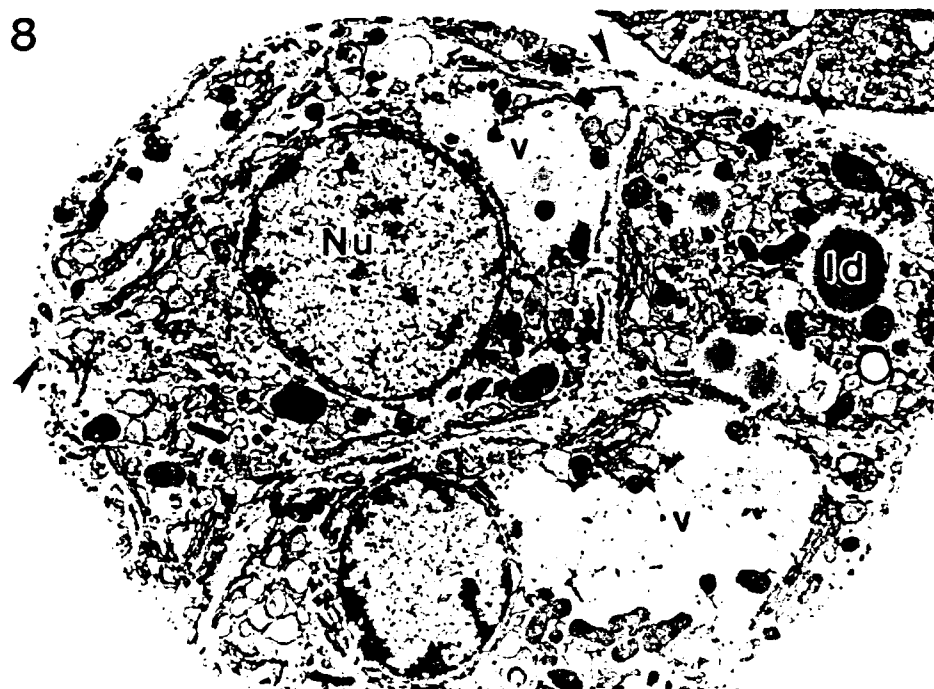
SEM studies of the organs or tissues revealed chords of elongated, lobular cells adhering to the tracheae associated with these organs, presumably the diffuse fat body (Fig. 3). These cells did not detach, nor was there any evidence that they proliferated during the incubation period. Attempts to dislodge them with trypsin solutions were unsuccessful.

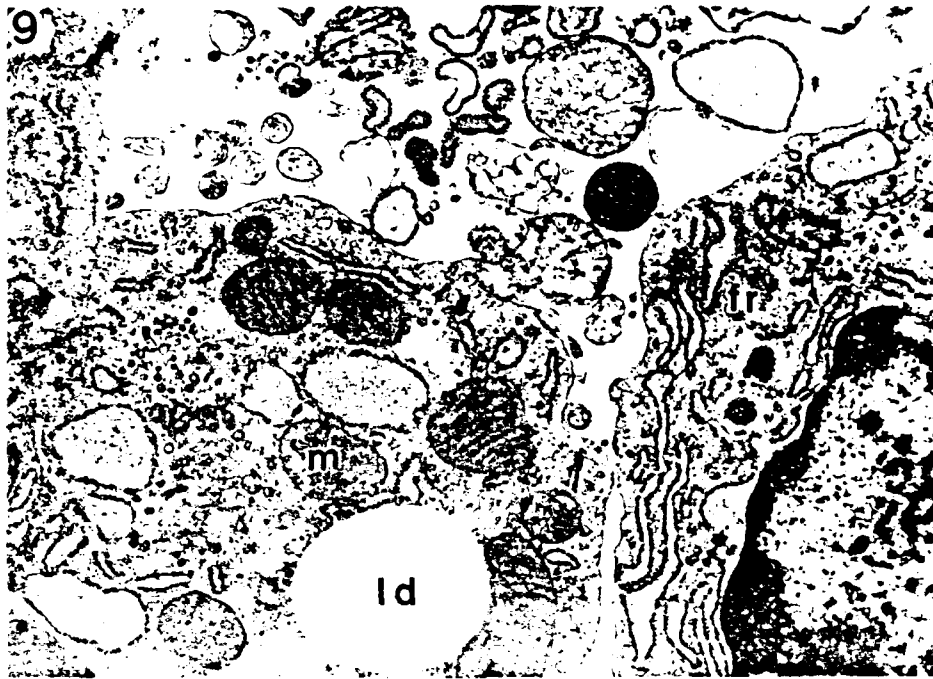
The origin and identity of the cells that proliferated during organ incubation or used for initiation of primary cultures is unknown. TEM studies (Fig. 4-6) of these cells revealed electron lucent or slightly electron dense inclusion bodies suggestive of lipid droplets; rarely, electron-dense inclusions were observed. Rough endoplasmic reticulum was also evident and in some cells it was extremely abundant. Comparison with transmission electron micrographs of cells of the established cell line RML-19 and the embryonic cell culture revealed many similarities. Cells from both the primary cultures and the RML-19 cell line also had inclusion bodies characteristic of lipid droplets and accumulations of rough endoplasmic reticulum. No evidence of extensive vacuolation, canaliculi, or membrane invaginations characteristic of secretory processes was observed.

TEM studies (Fig. 7-9) of cross sections of the fat body adjacent to the tracheae revealed cells with numerous mitochondria, abundant channels (canaliculi), and rough endoplasmic reticulum. The rough endoplasmic reticulum included many dilated as well as lamellate profiles. It contained a fine granular material characteristic of cells with high synthetic activity. Material of unknown chemical content also appeared to have been leached from these cells, leaving areas with numerous tiny vacuole like spaces









(Fig. 7, 8). In some cells, the plasma membrane appeared highly infolded, producing a greatly enlarged surface area characteristic of some type of secretory activity. In other cells, abundant accumulations of free ribosomes or polysomes, often were evident (Fig. 9). The entire tissue was surrounded by a basal lamina. No distinct division into different cell types was apparent.

Discussion

The results suggest that some tissue or cell type common to all of the organs or tissues used in this study is responsible for the production of ecdysteroids in vitro. A tissue that meets this criterion is the fat body. Fat-body cells are found in chords adhering to the tracheae throughout the tick's body, as well as on the outer surfaces of many internal organs. Consequently, this tissue will always be included when a body organ is excised and incubated. When the excised organs used in these studies were removed from the cultures, no further production of ecdysteroids was observed. These findings may implicate the diffuse fat body as the source of the in vitro production of immunoreactive substances. However, hemolymph, another diffuse tissue which might serve as a source of these hormones (Wigglesworth, 1979), can not be excluded. Hemolymph coagulates around organs excised during dissection. Although no in vitro production of ecdysteroids was observed in cultures initiated with hemolymph, fully differentiated hemocytes may have been removed when the culture medium was replaced. Whether tick hemocytes can produce ecdysteroids is unknown. However, it is doubtful that hemocyte contamination alone would have accounted for the in vitro ecdysteroid production observed in small organs such as the synganglion/lateral organ complex (ca. 20 ug).

Considerable variability was observed in the amounts of ecdysteroid produced in vitro. However, this is not surprising if fat-body is the source of ecdysteroid production in these culture environments, since the amounts of fat-body tissue removed at the time of excision of the various organs was unknown.

It is not surprising that D. variabilis cells proliferated in vitro. Yunker et al. (1984) noted that this was relatively common with tissues excised from this species. However, these cells do not appear to be responsible for ecdysteroid synthesis or secretion. Ecdysteroid production terminated following the removal of the excised organs (thereby removing the fat-body tissues), and no ecdysteroid production was detected in the primary cultures initiated from excised tick organs. Similarly, no ecdysteroid production was found in the EC culture or in the RML-19 cell line. Comparison of the ultrastructural characteristics of these cells from the primary cultures initiated from the partial fed D. variabilis females indicated resemblance with cultured cell lines. However, none of these cells appeared to have features characteristic of steroid secreting cells. According to Hagedorn (1985), "steroid secreting glands need large amounts of free ribosomes or polysomes and mitochondria." Additional features of steroid secreting cells, e.g., numerous lacunae, canaliculi, vacuoles or other intracellular cavities (Hagedorn 1985), were not seen in the tick cell cultures.

Our results parallel those of Ellis & Obenchain (1984). Those authors also observed the occurrence of ecdysteroids in medium supporting excised tick tissues. In one instance, they estimated as much as 25-30 pg ecdysteroid/ul of culture medium, although much lower amounts were recorded at other times. We also observed substantial changes in the concentration of

ecdysteroids in the medium during incubation. These variations may be related to differences in the amount of tissue incubated. If fat body was the source of the ecdysteroids released into the medium, the amount of this tissue would have varied greatly and, because of its diffuse nature, could not be measured.

The most common site of ecdysone synthesis in adult insects is the ovary, although some evidence implicates the oenocytes as a 2nd potential source of this hormone (Hagedorn 1985). In mosquitoes, fat body converts ecdysone to 20-OH ecdysone. In *Drosophila*, ecdysteroid is produced by unknown abdominal cells, possibly oenocytes, and this hormone acts on fat body to stimulate production of yolk protein (vitellogenins) (Hagedorn 1985). Insect fat body is not known to serve as the site of new ecdysteroid synthesis. Moreover, the fat body cells do not resemble insect steroid-secreting cells, which appear to need large amounts of free ribosomes or polysomes and mitochondria (Hagedorn 1985). Another possible source of ecdysteroid production in insects are the plasmatocytes, which are phagocytic amoebocytes of great diversity (Wigglesworth 1979). Wigglesworth suggests that the plasmatocytes may be a major site for conversion of ecdysone to 20-OH ecdysone. He also discounts Koolman's (1976) suggestion that the fat body is the chief site for this conversion, noting that hemocytes are closely associated with all tissues. In contrast to the plasmatocytes, the oenocytoids are believed to be responsible for the synthesis and transfer of lipids and phenols to the epidermal cells needed for the molting process, taking up these materials from the oenocytes and then attaching themselves to the epidermal cells where the transfer is made (Wigglesworth 1979).

In insects the fat body is a discrete tissue, comprising a single cell type, which serves as an important nutrient-processing and storage organ (Riddiford 1985). In ticks, however, the fat body is an exceptionally diffuse tissue that occurs along the tracheae and on the outer surfaces of most internal organs. Two major types of fat-body cells have been described in argasid and ixodid ticks on the basis of their staining characteristics with histochemical stains. Acidophils contain large lipid droplets that react strongly with Sudan dyes, while basophils lack these droplets (Obenchain & Oliver 1973). Close spatial relationships are reported between the fat-body cells and circulating as well as noncirculating hemocytes. In contrast with the insects, little is known about the function of tick fat-body tissues. Obenchain & Oliver (1973) suggest that their function is similar to that of insect fat-body. Chinzei & Yano (1985) reported vitellogenin synthesis by these tissues, providing further evidence of similarity with insects. Therefore, our findings indicating in vitro production of ecdysteroids by fat-body may indicate the ability of these tick tissues to metabolize ecdysone, e.g., to 20-OH ecdysone. Alternatively, these findings may indicate fat-body synthesis and metabolism of these compounds. Ellis & Obenchain (1984) suggested that "mixed tissues associated with the fat body" were producing ecdysteroids, singling out the basophilic cell (nephrocyte) as the most likely source.

The possible role of hemolymph as the source of edcysteroid production will be considered in future investigations.

Table 1. In vitro production of immunoreactive material by organs or tissues maintained in culture as determined by radioimmunoassay (RIA)*,**,***. Numbers represent picograms ecdysone equivalents-/100 ul of incubation medium (Exp. 1).

Synganglion															
lateral nerve															
plexus															
Ovary															
Salivary gland															
tracheae/															
fat-body															
Midgut															
Days in culture	pg/			pg/			pg/			pg/			pg/		
	Obs	exp	tick	Obs	exp	tick	Obs	exp	tick	Obs	exp	tick	Obs	exp	tick
1	42	--	16.8	132	--	52.8	68	--	27.2	36	--	14.4	713	--	475.3
3	0	32	0.0	154	99	61.6	92	51	36.8	94	27	37.6	479	535	319.3
5	0	24	0.0	224	74	89.6	173	38	69.2	96	20	38.4	412	401	274.7
7	194	18	77.6	441	56	176.4	176	29	70.4	92	15	36.8	811	300	540.7

* 50-ul aliquots were removed on each of the 4 observation days for assay (RIA). This amount was replaced with fresh medium to maintain a constant volume of culture medium.

** Amounts of tissue used/well were as follows: (1) synganglion/lateral nerve plexus, 0.102 mg; (2) ovary, 2.15 mg; (3) salivary glands, 4.50 mg; (4) tracheal trunks/fat tissues, 2.45 mg; (5) midgut, 21.72 mg. Ten partially fed virgin females were used as the source for the first 4 organs or tissues; 6 females were used to provide the midgut samples.

*** Controls, with medium only, were negative.

Table 2. In vitro production of immunoreactive substances by organs or tissues maintained in culture as determined by radioimmunoassay (RIA)*,** Numbers represent picograms ecdysone equivalent/100 ul of incubation medium (Exp. 2).***, +.

Days in culture	Synganglion											
	lateral nerve		Foveal		Ovary		Salivary		Tracheae/		Midgut	
	plexus		gland				gland		fat-body			
	Obs.	Exp	Obs.	Exp	Obs.	exp	Obs.	Exp	Obs.	Exp	Obs.	Exp
1	180	--	17	---	14	--	23	--	17	--	17	--
3	131	144	19	14	21	11	19	18	56	14	19	14
6	103	115	25	11	18	9	101	15	41	11	30	11
8	137	92	34	9	31	7	30	12	215	9	105	9
10	244	74	76	7	45	6	51	9	45	7	156	7
13	250	59	50	6	58	5	59	8	73	6	198	6

* 100-ul aliquots were removed on each of the 6 observation days for assay (RIA) and this volume was replaced with fresh medium.

** Each organ or tissue was incubated in 500 ul of Yunker-Meibos medium per well. The same number of ticks was used to provide organs or tissues and the same amounts were used as in Table 1.

*** 50-ul = 1 tick equivalent (T.E.) except for midgut (83-ul = 1 T.E.)

+ Controls, with medium only, were negative.

VI. EFFECTS OF ECDYSTEROID ANALOGUES ON DEVELOPMENT AND SEX PHEROMONE ACTIVITY IN TICKS

Introduction

Solomon et al. (1982) concluded that there was little cause for optimism regarding efforts to adapt knowledge of insect and acarine endocrinology to control of ticks. However, more recent studies have demonstrated that ecdysteroid analogues, particularly the triol analogue 22, 25-dideoxyecdysone (DDE) (Connat et al. 1983) and BSEA-28 (beta, 5-beta-14- alpha trihydroxy-5-beta-cholest-7-en-one) (Jaffe et al. 1983) exhibited biological activity when administered to ticks, in minute quantities. DDE was found to induce molting in soft ticks, Ornithodoros moubata, in doses as low as 35 ng/ml of blood; it induced substantial mortality when the concentration was increased to 500 ng/ml. When BSEA-28 was administered to laboratory rabbits by means of controlled release implant devices (Jaffe et al. 1983), we observed highly significant reductions in the time required for ecdysis, reduced body weight, and excitation of sex pheromone activity in camel ticks, Hyalomma dromedarii, fed on the treated animals.

The ability of these triol analogues to affect the same biological parameters regulated by natural ecdysteroid hormones suggested receptor recognition of these man made artifacts. Clearly, an opportunity for altering important biological functions is presented by this recognition. Consequently, we were interested in examining these effects further. The measurement of sex pheromone activity provided a convenient assay, since this function has been found previously to be regulated by ecdysteroids (Dees et al. 1984a).

This report summarizes the results of studies of the effects of administration of the triol analogue DDE via the implant technique described previously on sex pheromone activity and other biological functions in the camel tick, H. dromedarii.

Materials and Methods

Ticks. D. variabilis was colonized and reared as described previously (Sonenshine et al 1977). H. dromedarii were from a colony from the U.S. NAMRU-3, Medical Zoology Department, Cairo, Egypt (HH No. 59723, U.S. APHIS license no. 9433) and reared as described by Dees et al 1984a). Unfed adult females were of uniform age (2-3 weeks post-molting) and were held in an AMINCO-AIRE controlled environment chamber at 27+1° and 90+ 2% RH prior to feeding. These mature adult female ticks were allowed to feed 7 days on albino rabbits (Oryctolagus cuniculus), forcibly detached and collected for dissection.

Materials and Chemicals. Reservoir devices were made of poly-E caprolactone extruded as tubing, acquired and prepared as described previously (Jaffe et al 1986). The ecdysteroid triol, 22, 25-dideoxyecdysone (2 B, 3 B, 14 a-trihydroxy-5B-cholest-7-en-6-one) was synthesized by published methods (Thompson et al 1971) and was donated by Dr. Malcom Thompson, Laboratory of Insect Physiology, U.S. Department of Agriculture, Beltsville, MD. The active ingredient (AI), hereafter termed triol, was dissolved in caproic acid and the resulting solution deposited in the poly-E caprolactone tubes. The filled tubes were cut into 4-cm lengths and sealed with silastic plugs. The resulting solution filled 4-cm tubes each contained an estimated 45.3 mg

AI. Other poly-E caprolactone tubes were filled with propylene glycol cut into similar lengths, sealed, and were used as placebo controls.

Implantation of reservoir devices into host animals Implantation of the reservoir devices containing the different chemicals was done as described previously (Jaffe et al. 1986). All of the host animals were adult laboratory rabbits, O. cuniculus, that were never previously exposed to ticks (i.e., immunologically naive). The treated animals consisted of 3 females and 1 male; weights ranged from 2.2 to 3.7 kg/animal. Five to seven reservoir devices were implanted into the dorsal region of each animal and the incisions sealed with surgical sutures. The implants provided from 227 to 317 mg AI/rabbit (from 77.7 to 125.9 mg AI/Kg body weight. Controls included 3 animals, 1 male and 2 females, ranging in weight from 2.7 to 4.2 kg/animal. One animal received reservoirs containing only propylene glycol (pg), the second, reservoirs containing only tricaproin (both serving as placebo controls), while the third remained untreated. The treated animals were held one week for observation and monitored for evidence infection or disturbance of the sutures by the host.

Infestation. One week after implantation, each rabbit was infested with ca. 2000 H. dromedarii larvae. Observations of the progress of the infestation, recovery of engorged nymphs, mortality and/or molting were all done as described previously (Jaffe et al. 1986).

Direct application of 22, 25-dideoxycycdysone. In addition to administration of the triol by the controlled release method described above, triol was also administered to ticks by direct methods. Triol was dissolved in a mixture of DMSO:vegetable oil, 1:10, v/v, and 2 ul aliquots of solution containing 1 ug or 5 ug inoculated directly into engorged nymphs on the day of drop off. A total of 50 nymphs received 1 ug/nymphs; 50 others received

5 ug/nymph. Controls received solvent only. In addition, the triol was dissolved in a mixture of DMSO:acetone, 1:4, v/v, the concentrations of the AI adjusted to 25, 5, 1, and 0.25 ug/ul and 1 ul aliquots applied topically to engorged nymphs on the day drop off. Following treatment, the nymphs were examined daily for mortality or molting. Controls consisted of groups of 50 nymphs treated with 1 ul aliquots of DMSO, acetone, DMSO:acetone, 1:4, v/v, per nymph, or were untreated.

Maintenance of sex pheromone glands in vitro. Foveal glands were excised from partially engorged and from freshly molted, unfed H. dromedarii females, 24 and 48 hrs after emergence. The tissues were washed in Shen's saline (Oliver 1972) in a sterile hood with appropriate sterile technique, transferred to Yunker-Meibos culture medium (Yunker and Meibos, 1979), and maintained in culture as described previously. Foveal glands were placed in separate culture dishes, 20 gland pairs/dish. 20-hydroxyecdysone (20-OH ecdysone) and dopamine added to the wells containing glands from the fed ticks as follows: (1) 20-OH ecdysone, 10^{-4} M; (2) 20-OH ecdysone and dopamine, each 10^{-4} M; (3) no treatment (control). In addition, the synganglion and the foveal glands were left intact in the bodies of 20 other ticks, after the midgut, salivary glands, and other unrelated organs or tissues were removed, and these preparations were incubated in a fourth culture well. When all 4 preparations were completed, aliquots of a saline solution containing 5 uCi of ^{36}Cl labelled NaCl, was added to each culture well. The tissues were incubated for 48 hrs. The experiment was repeated 2 X. Assay for ^{36}Cl incorporation was done by liquid scintillation counting. For the experiment with glands from unfed females, stimulation was done with 20-OH ecdysone and 20-OH ecdysone and dopamine, and 22,25-dideoxyecdysone (22,25-DDE) as shown in Table 6. No ^{36}Cl NaCl was used in this experiment.

Sex pheromone activity. Adult H. dromedarii emerging from nymphs that engorged on the triol treated rabbits were separated into males and females, extracted with hexane and the sex pheromone, 2,6-dichlorophenol (2,6-dcp) separated with the aid of Florasil Sep Paks (Waters Assoc., Inc., Milford, MA) as described by Sonenshine et al (1982). The extracts were analyzed for evidence of 2,6-dcp by gas chromatography and electron capture detection, as described previously (Sonenshine et al. 1982), but with a Supelco SE-54 capillary column, 30 m x 0.25 mm I.D., split ratio 99:1 (Supelco, Inc., Bellefonte, PA) instead of the packed column described previously.

Results

Effects of hormone analogue on H. dromedarii feeding and molting. No deleterious effects were observed when treated rabbits infested with H. dromedarii larvae and nymphs were examined. Both life stages engorged readily and large numbers of replete nymphs were recovered from all of the triol treated animals (Table 3). The total numbers of engorged nymphs, 526 and 445 recovered from each of the 2 triol treated rabbits, respectively, were only slightly less than the total number recovered from the untreated control, 632. The mean weights for replete nymphal populations from the two triol treated rabbits, 20.4 +1.5 and 21.6 2.9 mg, respectively, were not significantly different than the mean weight of the engorged nymphs from the untreated control, 20.53 + 3.94 mg/tick ($t=0.27$, $p<0.5$, n.s., and $t=0.10$, $p>0.8$, n.s. However, survival of the nymphs from the treated rabbits was substantially reduced; 37% died without molting as compared to only 0.3% mortality in the nymphs from the untreated controls. The length of the ecdysial period was shortened slightly by feeding on the triol treated

rabbits, 18.5 ± 0.19 days ($t < 0.01$, 902 d.f.) When one compares the time when mortality occurred versus the time that molting occurred, it is apparent that mortality occurred very early, with peak mortality on day 3 post-engorgement; 27.2% of the engorged nymphs died within the first 5 days. Molting of the survivors resembles a unimodal curve, with the peak at 18 days.

When the tests were repeated (rabbits 3 and 4), ticks molting from one of the two animals exhibited exceptionally high mortality (48%) and greatly reduced mean body weights, while those molting from the other animals did not appear to have been affected.

Table 4 summarizes the results of the hormone implants on sex pheromone activity in adult camel ticks emerging from engorged nymphs fed on the 22,25-activity in adult camel ticks emerging from engorged nymphs fed on the 22,25 DDE treated animals. Comparisons are also made with previous studies using BSEA-28, 20-OH ecdysone, or direct inoculation of various ecdysteroids, and controls. The adults from the 22, 25-DDE treated rabbits appear to have produced the highest concentrations of sex pheromone, 2,6-DCP ever recorded. In addition to the very high levels observed in the females, high concentrations were also found in the males.

Table 5 summarizes the results of studies to determine the effects of hormonal stimulants on in vitro production of the sex pheromone, 2,6-DCP. Pheromone glands incubated in the presence of 20-OH ecdysone did not show any increase in 2,6-DCP content when compared with the controls (foveal gland only). However, pheromone glands incubated in the presence of both 20-OH ecdysone and dopamine did show increased 2,6-DCP content, ca. 2 X the control. Pheromone glands which remained attached to the synganglion also showed an increase in 2,6-DCP content. No incorporation of ^{36}Cl was found

when the organic fraction containing 2,6-DCP was assayed for radioactivity.

The experiment was repeated using pheromone glands excised from freshly molted females (within 1 or 2 days of emergence) (Table 6). Glands from both male and female adults were used. Pheromone glands incubated without hormones showed only trace amounts of 2,6-DCP. However, glands incubated in the presence of 20-OH ecdysone showed increased concentrations of pheromone. 20-OH ecdysone + dopamine was stimulatory for female glands, but not for male glands. The triol analogue, 22,25-DDE was highly stimulatory; in excised female glands, it induced a 7.8 fold increase in 2,6-DCP when compared to the 48 hour controls.

Table 3. Effects of presence of the hormone analogue 22,25- dideoxyecdysone
in rabbit hosts of H. dromedarii immatures on survival and molting.

		Mean			Mean			
		weight			molting			
Animal		Am't	eng.	Total	No.	No.	%	period
No.	sex	Kg	AI	nymph	nymphs	nymphs	molting	(days)
			(mg)	(mg)	fed	died	molted	
1	female	2.1	273	20.4	526	186	340	64.6
								18.3
								±0.14
2	male	2.4	227	21.6	445	173	272	61.1
								18.6
								±0.2
3	female	3.3	273	16.6	3224	412	2629	81.6
								17.4
4	male	2.9	227	9.8	1507	798	723	48.0
								17.6
5	male	3.8	0	18.4	1790	182	1627	90.9
								17.5
								±0.12
6	male	2.7	0	18.3	634	2	632	99.7
								19.8
								±2.1

Table 4. 2,6-dichlorophenol content of unfed Hyalomma dromedarii females immatures to which ecdysteroids had been administered by various methods.

Treatment	FEMALES		MALES	
	No. in sample	2,6-DCP content	No. in sample	2,6-DCP content
20-OH ecdysone (engorged nymphs)*	68	13.1	26	2.0
20-OH ecdysone (engorged nymphs +JH III)**	71	9.7	43	3.5
20-OH ecdysone implant in host***	100	5.8	105	0.0
BSEA-28 implant in host***	49	10.1	45	5.0
22,25-DDE implant in host	75	39.1	75	11.3
Control (1% saline) (1% saline)	65	5.6	75	0.0
Control (none)	100	5.7	75	0.3

* 10^{-3} M solution of 20-OH ecdysone in Shen's saline inoculated into engorged nymphs.

** 20-OH ecdysone inoculated as described above; JH-III applied topically in acetone.

*** From Jaffe et al. (1986) J. Med. Entomol. 23: 000-000 (in press).

Table 5. 2,6-dichlorophenol content of partially fed female Hyalomma
dromedarii sex pheromone glands maintained in vitro following
stimulation¹.

Organs incubated*	Stimulation (Compounds added)**	2,6-DCP content (ng/gland pair)
Foveal gland alone	20-OH ecdysone	4.1
Foveal glands alone	20-OH ecdysone + dopamine	12.6
Foveal glands + synganglion and neural connections	None	16.6
Foveal glands alone	None (control)	6.7

* Incubated for 48 hrs in Yunker-Meibos culture medium.

** 10^{-4} M concentration.

Table 6. In vitro stimulation of sex attractant pheromone activity,
2,6-dichlorophenol, in excised foveal glands of the camel tick,
Hyalomma dromedarii*

<u>Treatment</u>	<u>nanograms 2,6-DCP/gland pair</u>	
	<u>Males</u>	<u>Females</u>
24 hr incubation**	0.53	0.72
24 hr incubation	2.43	2.57
+ 10 ⁻⁴ M 20-OH ecdysone**		
48 hr incubation	n/d	1.70
48 hr incubation	0.54	4.10
+ 10 ⁻⁴ 20-ecd/dopamine**		
48 hr incubation	1.52	13.20
+10 ⁻⁴ 22,25 DDE**		

* Extraction and analysis of 2,6-dichlorophenol as described by
Sonenshine et al (1982).

** Foveal glands dissected from surface sterilized virgin females
within 48 hr of molting and cultured in Yunker-Meibos medium.
(Please see text for details).

VII. JUVENILE HORMONE(S) IN TICKS: EVIDENCE FOR OCCURRENCE AND POSSIBLE ROLE IN SEX PHEROMONE ACTIVITY

Introduction

Although the occurrence of juvenile hormones/gonadotropic hormones is generally assumed to occur in virtually all arthropods, no direct evidence of their presence in ticks has been obtained to date. Ticks are undoubtedly an ancient group of arachnids (Hoogstraal, pers. commun.) and their regulatory systems may not merely mimic those of the better known insects. Gonadotropic activity in ticks is well known (Solomon et al 1982) and the gonadotropins may be similar or identical to the molecules that regulate development of the immatures, i.e., juvenile hormones (JH), as is the case in insects (references). Synganglia from fed-mated Ornithodoros moubata were found to induce oviposition in 41% of fed virgin females of this species (Aeschlimann, 1968). Other evidence of synganglion involvement in regulating oogenesis, all in argasid ticks, is summarized in recent reviews (Solomon et al 1982, Diehl et al 1982). Evidence implicating JH as the gonadotropin in ticks was described by Pound and Oliver (1979). These workers used authentic JH to restimulate oogenesis in soft ticks treated with the anti-allatotropin Precocene II. They hypothesized that this blocking agent removed the essential hormonal stimulus, whereupon the intervention of the exogenous JH "rescued" the ovary, restimulating the process. These and other findings reviewed elsewhere argue persuasively for but do not prove the existence of JH in ticks. Further progress is largely dependent upon identification of the specific hormone.

Although unequivocal identification of the hormone is the ideal solution to the problem of JH in ticks, the exceptionally minute quantities that may be expected to occur in these small arthropods makes this an unusually difficult goal to achieve. Studies done by J. Baehr at the Universite de Marie et Pierre Curie, Paris, France, revealed JH-3 at very low concentrations in the hemolymph of partially engorged virgin female ticks when hemolymph samples from these specimens were assayed by RIA, specifically, 3.4 ± 1.3 pg/female in D. variabilis hemolymph and 76.7 ± 3.1 pg/female in of JH-3 in H. dromedarii hemolymph. Although the RIA results suggest the probable presence of JH in these species, chemical methods are needed to confirm these findings. Important information may be obtained by study of JH enzymes and JH metabolites. The latter may be expected to occur in considerably greater quantities than the parent compound. In insects, the various JH types are degraded rapidly into the corresponding acids and diols by non-specific hemolymph esterases and JH specific esterases and epoxide hydrolase, and the products can accumulate in considerable quantity. Thus, measurement of the degradation of the authentic hormone and/or study of the resultant acid, acid/diol and diol metabolites can provide useful information as well as compelling evidence of natural JH. Photoaffinity assays are useful as a means of determining the presence of JH specific binding proteins. To perform this latter assay, collaboration was arranged with Dr. Glen Prestwich, Department of Chemistry, State University of New York at Stony Brook, NY. We collected hemolymph from engorged virgin and engorged mated females, preserved the hemolymph with specific reagents provided for this purpose, and forwarded these materials to Dr. Prestwich for assay.

We undertook studies to determine (1) whether enzymes were present in tick hemolymph capable of metabolizing authentic JH, (2) whether the end

products of this metabolic degradation were similar or identical to known JH acid, acid/diol, and diol metabolites that result from degradation of the corresponding hormone in insects, and, (3) whether JH specific binding proteins were present.

Materials and Methods

Ticks. The American dog tick, D. variabilis was colonized and reared as described previously (Sonenshine et al 1977). Immature ticks were allowed to feed on albino rats (*Rattus norvegicus*), adults on rabbits (*Oryctolagus cuniculus*). The camel tick, H. dromedarii was colonized from a stock originally from the U.S. NAMRU-3 Medical Zoology Department, Cairo, Egypt (HH No. 59723, U.S. APHIS license No. 9433) and reared as described previously (Dees et al 1984 b). Ticks were held in an AMINCO AIRE climate Lab environmental chamber at $27 \pm 0.5^{\circ}\text{C}$ and $90 \pm 2\%$ RH during their non-parasitic periods.

Chemicals and standards. All solvents, including those used for extraction (except ethanol), were HPLC grade (Burdick & Jackson, Muskeegan, MI). Hexane was double distilled (Omni-Solv, Krackler Chemical Co., Albany, NY). Dimethyl sulfoxide (DMSO) (spectranalyzed) and iso-octane were obtained from Fisher Scientific Products, Inc., Fairlawn, NJ. Ethyl oleate and the authentic standards JH1, JH2, and JH3 were from Sigma Chemical Co., St. Louis, MO, while radiolabelled ^3H JH was from New England Nuclear, Boston, MA (NEN). To purify the radiolabelled JH, it was dried (N_2), reconstituted in 100% ethanol, and chromatographed by TLC as described above. Tobacco hornworm (THW) larvae Manduca sexta were from Carolina Biological Supply Co., Burlington, NC. The esterase standard was porcine

liver extract, type I (Sigma). Acrylamide, tris, temed, Bromphenol Blue tracking dye (BB) and Commassie Brilliant Blue (CBB) for staining of proteins and BioLyte for isoelectric focusing were all from BioRad (Richmond, CA). Fast blue was obtained from Sigma.

Assays for JH by High Performance Liquid Chromatography (HPLC). Three types of tick materials were used for extracts, namely (1) hemolymph; (2) synganglia, all from part fed virgin females, fed 7 days (PFV females) or mated replete females (MR females); (3) whole body (WB) extracts. The hemolymph collections comprised 920 ul from PFV females and 750 ul from MR females; 150 synganglia were from PFV females. Extractions were made according to the method of Connat (1982), with modifications. Hemolymph was extracted with iso-octane. The synganglion or WB extracts were homogenized in cold acetonitrile (ACN) with the aid of celite, filtered (fritted glass funnel), and the filtrate re-extracted 3 X with hexane:water with 1% NaCl. The organic and aqueous phases were separated, concentrated (N_2), and separated further on either (1) Bakerflex IB2 silica gel TLC plates (Arthur H. Thomas, Inc., Philadelphia, PA) or reversed phase C-18 plates (J. T. Baker Co., Phillipsburg, NJ). Typical solvent systems were benzene:ethyl acetate, 96:4, v/v, for the silica gel plates and methanol:water, 80:20, v/v for the C-18 plates. The zone expected to contain the JH was eluted (hexane:ethyl acetate, 90:10), dried, and reconstituted in ACN. JH acids and diols were prepared by incubation of the authentic standards plus 3H labelled JH with THW hemolymph or acidification with HCL. The highly concentrated samples were assayed by HPLC, using a Waters Assoc. system (Milford, MA) consisting of a Model 441 UV fixed wavelength detector (214 nm filter), 2 Model 510 pumps, a U6K septumless injector, a Z-module to contain the column, a Model 721 Systems Controller, and a Model 730 Data-Module. The column was a

NovPak 5-um C-18 column, 81 um I.D. X 10 cm long (Waters). The solvents were ACN and water. Typical solvent ratios and pumping parameters were 75:25 and 1 ml/min, respectively for isocratic separations; a variety of gradient elutions were also used. Under these conditions, JH1, JH2, and JH3 eluted at 12.42, 6.75 and 8.96 min, respectively. The acid and diol of JH3 eluted at 4.08 and 7.69 min, respectively. The sensitivity of the system, at 0.005 AUFS, was ca 2 ng.

JH esterase activity. Hemolymph was collected from PFV females as described above and held at ca 0°C. A sample of 150 ul of fresh hemolymph was added to 6 ml of 0.1M phosphate buffer and inoculated with a dilute ethanolic solution containing 1 uCi of ³H JH-3 and 50 ug of non-labelled authentic JH-3. The mixture was incubated, with gentle shaking, for 60 min at 30°C. The reaction was stopped by addition of 250 ul of methanol:ammonia:water, 10:9:1, with vigorous shaking. Iso-octane, 15 ml, was added with vigorous shaking to extract the remaining JH and centrifuged 2 X (16,000 rpm, 2 min) to partition into organic and aqueous phases. The aqueous phase was separated, re-extracted with iso-octane, and the fractions combined. Emulsions at the interface of the 2 layers prevented complete separation. Controls consisted of (1) tick hemolymph + JH-3 and solvents, as described above, but inactivated by heating in a water bath at 80°C for 60 min, and (2) as described above, but without tick hemolymph. In addition, the same study was also done with hemolymph collected from tobacco hornworm larvae (TBH), with the same controls. Analysis of the extracts was done by radioassay with a Beckman model LS 250 liquid scintillation counter (estimated 37% efficiency for tritium). Aliquots of the aqueous and organic phases were also separated further by TLC and HPLC and eluates collected by these methods were assayed for radioactivity as described above.

Electrophoresis of tick esterases. A hemolymph sample containing 500 ul was collected from PFV females as described above and centrifuged for 10 min at 16,000 RPM to remove hemocytes and cellular debris. The supernatant was recovered and dialized (VWR Scientific Co., Phillipsburg, PA, size 8) to remove salts and small peptides. Controls were done using (1) TBH larvae, and (2) porcine liver extract, Type I. Protein determinations were done using the BioRad protein assay (BioRad, Richmond, CA). The total protein concentration of the tick hemolymph was 115 ug/ul; of the TBHL hemolymph, 56 ug/ul; of the porcine liver extract, 1 ug/ul. Molecular weight markers, high and low (BioRad) were used to determine the approximate size of the hemolymph proteins.

Polyacrylamide gels were prepared as column gels or slab gels. Column gels were prepared at a concentration of 5% with 0.5 M tris-HCL, Temed, and ammonium persulfate (pH 8.9). Following loading of the samples (200 to 400 ug) or the standards (10 to 20 ug) onto the gels, electrophoresis was done in a Buchler Model 1004 electrophoresis cell (Buchler Instrument Co., Fort Lee, NJ) with tris-glycine buffer (0.04M, pH 8.3) at 3 mA/tube or until the tracking dye (BB) reached the bottom of the gel. Staining for proteins was done with CBB, 0.25% in 50% methanol, followed by destaining overnight in methanol acetic acid (30%:7%). To analyze for esterases, the gels were prepared in 0.03 M borate buffer, pH 8.0. Following loading of the samples or standards, electrophoresis was done as described above, but a 0.3 M borate buffer was used and staining was done with Fast Blue (FB) esterase stain with 0.1% alpha-naphthol acetate in acetone as the substrate. Slab gels were prepared by applying 10% separating gel solutions to 100 X 125 mm glass plates. Following polymerization (1 hr), a 4.75% stacking gel was used to cover the first layer. Samples or standards were loaded in wells cut at the

top of each gel. Electrophoresis was done overnight in a BioRad Model 220 slab gel electrophoresis cell; the well buffer was 0.04 M tris-glycine (pH 8.3). Staining and destaining was done as described above.

For isoelectric focusing, slab gels with BioLyte 3-10 were prepared in accordance with the manufacturer's instructions. Samples were applied on 3 X 4 mm oval pads near the cathode and electrophoresis was continued until the voltage became constant. Staining and destaining was done as described above.

Effects of authentic JH on tick development. Following trials with a variety of solvents and techniques, three methods of administration were used, (1) inoculation, (2) topical application, and (3) by contact, in Petri dishes (Bowers et al. 1976; Leahy and Booth, 1980). All ticks were treated on the day of drop off. Inoculations were done with 2 ul of ethyl oleate containing different concentrations of JH compounds. The concentrations used were 1, 5, 10, 20, 50, and 100 ug/nymph. In addition, others were inoculated with 0.5 female equivalents (FE) of PFV female hemolymph, reconstituted in ethyl oleate. Controls were done with solvents only. Topical applications were done with different concentrations of JH-3 dissolved in DMSO:acetone, 1:4, v/v. and administered in 1 ul aliquots onto the venter of 50 engorged nymphs immobilized with tape. The concentrations used were 1, 5, 10, 20, 50 and 100 ug/nymph. Controls included nymphs treated with (1) solvents only, or (2) were untreated. To treat by the contact method, solutions of JH-3 dissolved in DMSO:acetone, 1:9, were applied to filter paper circles placed in glass Petri dishes, 9-cm diam, top and bottom. The total treated area was 127 cm². Nymphal ticks, 50/treatment, were released to each treated dish and allowed to contact the treated surfaces for 3 hr. The concentrations used were 0.1, 0.5, 1, 2, 5 and 10 ng/cm². Controls included

(1) nymphs exposed to solvents only and (2) untreated nymphs. The treated ticks in all 3 treatment types were monitored daily for mortality and time of molting.

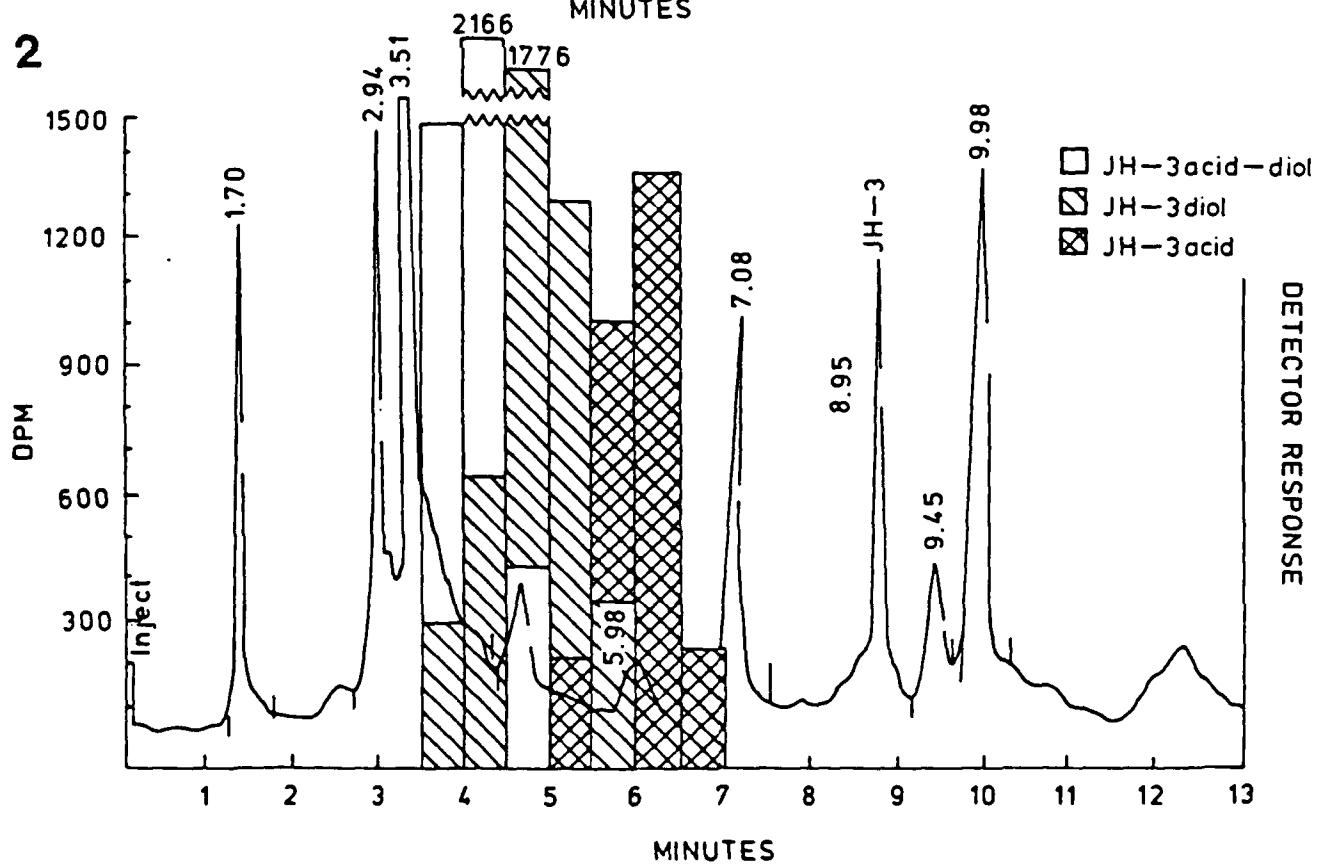
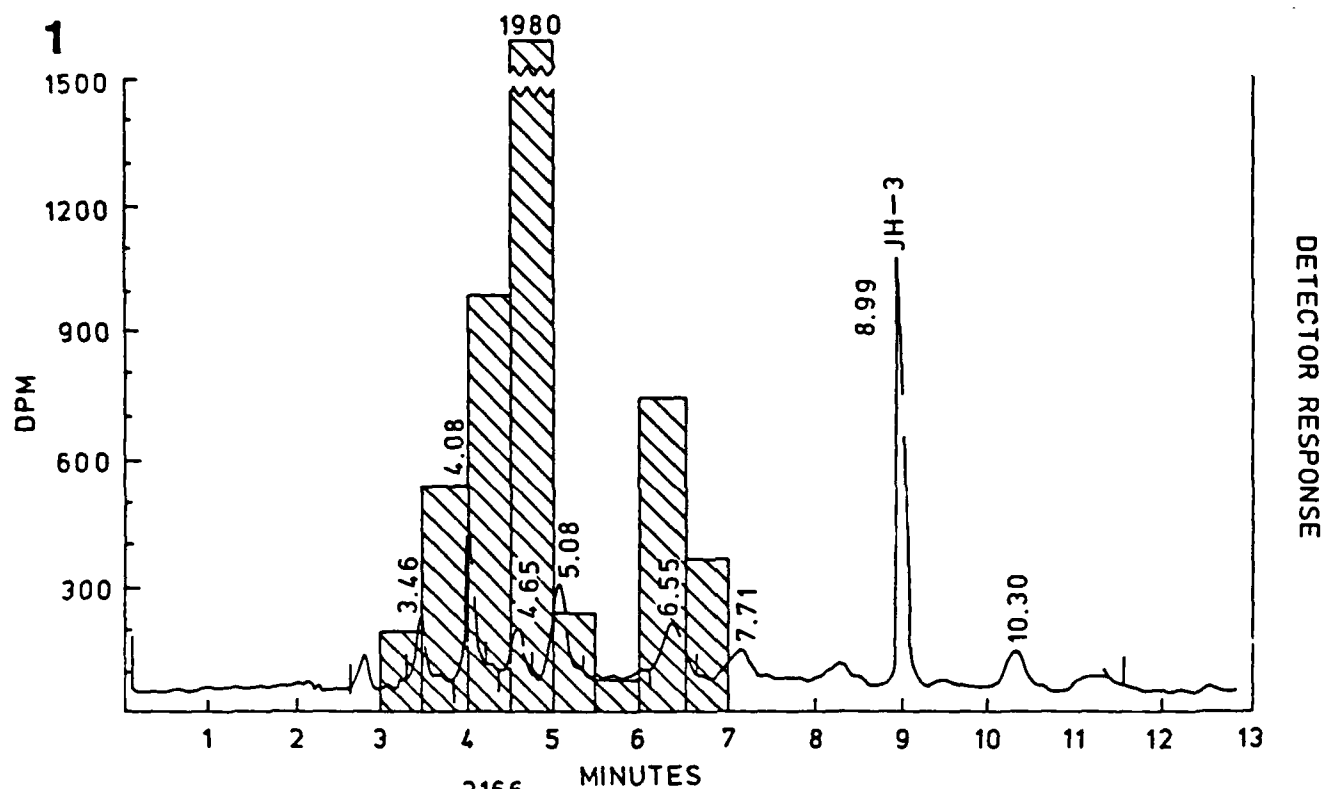
Results

Incubating radiolabelled juvenile hormone with tick hemolymph resulted in hydrolysis of this compound, with most of the polar end products appearing in the aqueous phase. Table 7 summarizes the results of a series of experiments. At the completion of the incubation, an average of 69.4% was found in the aqueous phase, vs an average of only 30.6% remaining in the organic phase. In contrast, almost all of the JH-3 remained in the organic phase following incubation with inactivated tick hemolymph (average of 90.6%) or Shen's saline without hemolymph (average of 95.5%). Similar results were obtained when juvenile hormone was incubated with THW larval hemolymph (Table 8). When an aliquot of the aqueous phases from the active hemolymph incubation was chromatographed on a reversed phase (C-18) plate (Table 9), 19.9 and 39.9% of the radioactivity was found at 6 and 7 cm from the origin, respectively, indicating that most of the radioactivity was in the form of compounds more polar than JH-3, presumably JH acid, acid/diol, and diol metabolites. Chromatographing an aliquot of the organic phase from this same incubation revealed only 25.0 of the radioactivity co-eluting with JH-3, i.e., the radiolabelled JH-3 remaining from the original treatment. Very different results were obtained when the aqueous and organic phases from the controls (inactivated hemolymph or no hemolymph) were chromatographed on C-18 plates; almost all of the radioactivity, 86.3% and 94.1%, co-eluted with JH-3. The same general trends were obtained when D.

variabilis PFV female hemolymph was used (Table 10). However, considerably greater amounts of undigested JH-3 remained than was found with the THW larval hemolymph, with a major peak, representing 56.1% of the sample radioactivity, co-chromatographing with JH-3. Only one major polar peak of radioactivity, representing 14.5% of the sample radioactivity, was found in the form of highly polar compounds. The total radioactivity recovered from the digests and from the TLC plates was only 46.5% of the total introduced at the start of the experiment, largely due to emulsions that formed at the interface between the 2 phases. Also of interest in the case of the tick hemolymph digests was the presence of weakly active spots at 3 and 4 cm, representing compounds more polar than JH, but not as polar as the much more prominent fraction at 7 - 8 cm.

HPLC of the aqueous phase eluted from the TLC plate (6-7 cm zone) revealed 3 peaks that were radioactive (Fig. 1). Together, these 3 fractions accounted for 14.3%, 49.6%, and 17.0% of the total radioactivity of the sample, respectively. All 3 peaks co-eluted with authentic ^3H labelled JH-3 metabolites donated by Dr. M. Rowe, Department of Entomology, North Carolina State University, Raleigh, NC (Fig. 2). Fraction 1 coelutes with the acid, fraction 2 coelutes with the acid/diol, and fraction 3 coelutes with the diol. HPLC of a sample of (500 ul) of D. variabilis PFV female hemolymph also revealed the same peaks.

We also observed evidence of hemolymph esterases in feeding females by column gel electrophoresis (SDS-PAGE). Using Fast Blue esterase specific staining, we observed 4 distinct esterase bands in D. variabilis hemolymph. Based on the use of molecular weight markers, the estimated weights of the proteins were 218,000, 205,000, 191,000 and 118,000 daltons. In contrast with the ticks, only 3 esterase bands were observed in THW larval hemolymph.



When these same materials were analyzed by isoelectric focusing on a slab gel, 10 of 16 hemolymph proteins were found to be esterases, including three that were intensely reactive. The concentrations of tick hemolymph esterases also appeared to be much greater than in the tobacco hornworm larval hemolymph.

Treatment of H. dromedarii nymphs by the topical method had no apparent affect on the duration of the ecdysial period; the means for the different treatment groups were remarkably uniform, from 19.3 to 20.0 days. Survival was unaffected by the lower dose treatments. However, substantially reduced survival occurred with the 50 and 100 ng treatments. No higher doses were administered, and it is not known whether this represents a dose response curve or not.

Treatment of H. dromedarii nymphs by the contact method did not appear to have any effects on the ecdysial period or survival of the treated ticks. The ecdysial periods ranged from 17.1 days at 0.5 ng/cm² to 21.5 days at 10 ng/cm² treatments. However, the duration of the ecdysial period was just as long in the case of the nymphs exposed to 0.1 ng/cm², the lowest dose used, as at 10 ng/cm², the highest doses, and there was no evidence of a dose response curve. Survival remained high in most treated groups except those exposed to 0.1 ng/cm², the lowest dose used.

Tests with radioactive JH-III demonstrated that 22.7% of the compound administered topically in DMSO;acetone enters the body of the treated ticks.

Discussion

The results demonstrate that tick hemolymph has an impressive ability

to enzymatically degrade exogenous JH. Non-specific carboxy-esterases are probably responsible for much of this metabolism of JH, resulting in the acid metabolite of the corresponding parent molecule. However, the presence of two other metabolites strongly implicates the presence of a more specific enzyme capable of hydrolyzing the epoxide bond, i.e., a specific epoxide hydrolase. The presence of 3 metabolites, thereby implicating a JH-specific enzyme, strongly implicates the presence of JH itself.

Tick hemolymph is very similar to tobacco hornworm larval hemolymph with regard to its ability to metabolize JH. Tick hemolymph contains numerous proteins with esterase activity which are especially evident when they are separated by iso-electric focusing; at least 10 esterase bands are evident by this technique, including 3 with especially intense activity.

In insects, JH inactivation is believed to begin in the hemolymph, where the free hormone is attacked by non-specific carboxyesterases and degraded to the corresponding acid. However, much JH is protected by JH specific binding proteins, and survives intact unless JH specific esterases capable of reacting with the bound hormone also occur (Riddiford & Truman, 1978). Further breakdown to the diol and acid/diol forms is accomplished by membrane bound enzymes, especially by epoxide hydrolase. In insects, this enzyme is found primarily in the fat body, in the epidermis and, to a lesser extent, in other tissues. Protein bound JH is readily susceptible to attack by these intracellular enzymes. The various JH metabolites that result no longer bind to the carrier proteins and are excreted intact or as sulfate conjugates (Riddiford & Truman, 1978).

The results of the photoaffinity assay for the presence of specific JH binding protein were also positive. The tests will be repeated when sufficient additional hemolymph has been accumulated, and precise values for the

amounts of JH-binding protein will be calculated.

Exogenous JH may induce a variety of physiological effects in ticks. Pound and Oliver (1979) observed restoration of ovarian activity by JH administered after treatment of female Ornithodoros parkeri with the anti-allatotropin Precocene-II. More recently, Connat et al demonstrated excitation of vitellogenesis in fed virgin O. moubata which normally do not oviposit unless mated. The most convincing evidence of JH as a gonadotropic /vitellogenic hormone has been obtained with argasid ticks (although Bassal and Roshdy (1974) were unable to induce such action by JH in Argas arboreus). The role of JH in ixodid ticks is much more difficult to discern. Ioffe and Uspenskiy (1979) observed that Altozar, a JH analogue, delayed molting in non-diapausing Ixodes ricinus, but accelerated molting in diapausing nymphs, i.e., disrupted; diapause. In their review of the literature on this subject, Solomon et al. (1982) noted that virtually all studies with exogenously administered JH in ixodid ticks reported no discernible juvenilizing effects. The sterilizing effects obtained with massive doses of Precocene-II in Rhipicephalus sanguineus (Leahy & Booth, 1980) may represent toxic rather than true hormonal effects. Moreover, true extension of the ecdysial period was not observed by these authors, who noted that treated ticks failed to molt well beyond the expected, ecdysial period, but did not differentiate between delayed mortality and delayed ecdysis. Presumably, JH exists in ixodid ticks, affecting developmental processes as well as regulating spermatogenesis, vitellogenesis and oviposition in the adults. However, proof of the existence of this elusive hormone and its actions remains to be discovered.

At the present stage of our study, we have been able to characterize the rapid degradation of JH by tick hemolymph to 3 different metabolites

and, by means of the photoaffinity assay, the presence of JH-binding protein. These findings provide new evidence of the existence of JH in adult female ticks where it may serve as a gonadotropic hormone. They also suggest that further study using definitive chemical techniques (especially GC/MS) may conclusively demonstrate the existence of this elusive molecule in these arthropods.

Table 7. Metabolism of juvenile hormone by hemolymph from part fed
Dermacentor variabilis females (expressed as percent of amount
administered found in aqueous or organic phase)

Trial No.	ACTIVE HEMOLYMPH		INACTIVATED HEMOLYMPH		NO HEMOLYMPH	
	Aqueous	Organic	Aqueous	Organic	Aqueous	Organic
1	67.0	33.0	6.4	93.6	8.7	91.2
2	82.5	17.5	17.5	82.5	1.6	98.4
3	58.6	41.4	4.4	95.6	3.2	96.8
Avg.	69.4	30.6	9.4	90.6	4.5	95.5

Table 8. Metabolism of juvenile hormone by hemolymph from Tobacco hornworm
Manduca exta larval hemolymph (expressed as percent of amount
administered found in aqueous or organic phase)

PHASE	ACTIVE	INACTIVE	NO HEMOLYMPH
Aqueous	77.5	10.7	1.8
Organic	22.5	89.3	98.2

Table 9. Metabolism of JH-3 by Tobacco hornworm (Manduca sexta)
hemolymph;^{1/} percentage of radioactivity eluted from each zone
separated by TLC.

cm from origin	<u>ACTIVE HEMOLYMPH</u>		<u>INACTIVE HEMOLYMPH</u>		<u>NO HEMOLYMPH</u>	
	aqueous	organic	aqueous	organic	aqueous	organic
0 - 1	0.0	0.7	0.0	0.1	0.0	0.2
1 - 2	0.0	2.1	0.6	0.5	0.0	0.5
2 - 3 ^{2/}	2.1	25.0	5.8	86.3	1.7	94.1
3 - 4	1.3	0.5	4.1	0.0	0.3	0.0
4 - 5	0.0	0.5	0.4	0.5	0.3	1.5
5 - 6	1.5	0.5	0.3	0.3	0.2	0.3
6 - 7	19.9	0.0	0.3	0.1	0.2	0.4
7 - 8	39.9	0.0	0.2	0.1	0.2	0.2
8 - 9	2.6	2.0	0.0	0.0	0.0	0.0
9 - 10	1.3	0.0	0.0	0.0	0.0	0.0

^{1/} Done on C-18 plate and developed with methanol:water, 80:20 (v/v).

^{2/} JH-3 migrate to this zone.

Table 10. Metabolism of ^3H JH-3 by part-fed virgin female Dermacentor variabilis hemolymph;^{2/} percent of radioactivity eluted from percentage of radioactivity eluted from each zone separated by TLC.

cm from origin	ACTIVE HEMOLYMPH		INACTIVE HEMOLYMPH		NO HEMOLYMPH	
	aqueous	organic	aqueous	organic	aqueous	organic
0 - 1	1.2	0.5	0.0	0.3	0.1	0.7
1 - 2	1.1	0.5	0.2	0.6	0.1	0.7
2 - 3 ^{2/}	6.6	56.1	3.0	89.1	1.4	86.3
3 - 4	3.5	4.3	1.5	1.6	0.2	4.3
4 - 5	3.3	1.1	0.4	0.4	0.4	1.6
5 - 6	1.4	0.8	0.3	0.7	0.2	1.6
6 - 7	14.5	0.4	0.3	0.6	0.2	0.4
7 - 8	2.0	0.5	0.2	0.8	0.2	0.5
8 - 9	0.8	0.4	0.0	0.2	0.1	0.4
9 - 10	0.9	0.4	0.0	0.0	0.1	0.4

^{1/} Done on C-18 plate and developed with methanol:water, 80:20 (v/v).

^{2/} JH-3 migrates to this zone.

Table 11. Effects of treatment of *H. dromedarii* nymphs with JH-³
in DMSO:Acetone (1:9) administered by the contact method on
survival and the duration of ecdysis.

Days post- treatment	Student only	<u>Concentration of JH-III in ng/cm²</u>					
		0.1 ng	0.5 ng	1.0 ng	2.0 ng	5.0 ng	10.0 ng
9	0	0	0	1	0	0	0
10	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0
13	0	0	0	2	0	0	0
14	0	0	0	0	0	0	1
15	2	0	8	1	1	0	0
16	4	0	7	2	5	6	0
17	1	1	2	0	1	4	0
18	9	0	12	0	15	10	2
19	0	0	0	1	0	0	1
20	6	1	0	13	8	0	5
21	8	0	0	0	10	10	11
22	4	0	5	3	0	10	4
23	3	1	0	5	0	1	4
24	2	0	0	5	0	0	2
25	0	0	0	0	1	0	5
26	1	1	2	0	0	0	4
Total surviving	40	4	36	33	41	41	39
% mortality	20.0	92.0	18.0	34.0	18.0	18.0	22.0
Mean days ecdysis	19.8 ±2.7	21.5 ±3.9	17.1 ±4.3	20.1 ±3.6	18.9 ±1.8	19.2 ±2.9	21.5 ±4.4

VIII. HORMONAL REGULATION OF REPRODUCTION IN TICKS AND PARASITIC MITES

Introduction

Annual progress report on work completed at Georgia Southern College on ONR Project "Hormonal-Pheromonal Interrelationships in Ticks and Parasitic Mites," (Contract N00014-80-C-0546). James H. Oliver, Jr., Co-Principal Investigator (subcontractor).

Oocyte Maturation and Oviposition

Little is known about the hormonal control of oocyte maturation and oviposition in ticks. The lack of taomosis (organization of body into separate regions such as head, thorax, abdomen) and the extreme degree of fusion of nerve ganglia, etc. make selective ablations of tissues, ligations, etc. very difficult. The nature of the circulating hormones, site of vitellogenin synthesis, and the extract process of ovarian development are poorly understood.

In insects, juvenile hormone (JH) is known to function gonadotrophically in adults and is produced by the corpora allata (CA). Several reports suggest a role for JH-like compounds in the reproduction of ticks similar to that reported in insects. Two of these reports provide particularly strong evidence that a JH-like substance is necessary for egg development and oviposition in ticks (Pound and Oliver 1979; Connat et al. 1983).

1. An attempt to determine if JH plays a similar role in parasitic mites involved the use of the anti-JH compound precocene 2 (P2) applied to the chicken mite, Dermanyssus gallinae. Experiments were designed to determine if P2 would affect egg production, and if so, whether exogenous JH application would restore egg production. If application of JH to the mites restored egg production, this would be strong evidence for the natural presence and necessity of a JH-like compound in female mites and would lend

credence to the hypothesis that JH (or closely related compound) functions in a gonadotrophic manner in acarines similarly as in insects.

Two mg of P2 were exposed to fed female Dermanyssus gallinae for various lengths of time (24-96 h) and at several concentrations (0.25-4.0 mg). The P2 caused a reduction of progeny produced by the treated females. Reproductive capacity was significantly restored in P2 treated females by application of the insect juvenile hormone III (JH III), but was not restored to the full capacity of untreated control females. Although P2 reduces progeny production among treated females, and JH III partially restores it, the P2 doses tested were not strong enough to completely stop progeny production. Moreover, the doses of P2 used were not strong enough to permanently damage the tissue producing the putative JH-like gonadotrophic hormone as shown by the natural reproductive recovery of P2 treated females that were allowed additional feedings and recovery times. Results of this study suggest that a JH-like compound probably functions in egg development in mites.

2. Other experiments concerning the putative role of JH in egg maturation and oviposition involved the tick Ornithodoros parkeri. The purpose of these experiments was to determine the role of JH on egg maturation in fed-virgin females. Females of this species require a blood meal and mating for vitellogenesis to occur. Attempts were made to produce vitellogenesis in fed unmated female O. parkeri by topical applications and injections of JH and farnesol. Scoring of fed virgin female reproductive systems and fed mated female controls was done subsequent to treatment. Topical applications and injections of JH initiated vitellogenesis, but did not induce oviposition. Injection of JH stimulated vitellogenesis more than did topical application regardless of whether JHI, JHII or JHIII was used. The fact that JH stimulates vitellogenesis, but that oviposition did not occur,

allows several different interpretations. Perhaps JH is necessary early in the cycle of egg maturation, and some other compound(s) is necessary for later development and/or oviposition. Alternatively, perhaps the timing and concentrations of JH were not physiologically attuned to this species. Clearly, JH deteriorates rapidly and perhaps a pulse-type of JH treatment would more nearly simulate the normal condition. Preliminary experiments involving several catecholamines indicate that they may also play a role in stimulating vitellogenesis. More will be reported on these experiments in the next progress report.

3. Another series of experiments concerning stimulation of egg maturation and oviposition in O. parkeri involved hemocoelic injection and vaginal insertion of selected male reproductive and non-reproductive tissue homogenates into fed virgins. These treatments stimulated ovum maturation and/or oviposition to varying degrees. Mean times for oviposition and mean numbers of eggs laid per ovipositing female receiving hemocoelic injections of male reproductive tissue homogenates did not differ significantly from fed-mated controls. In addition, hemocoelic injection of male salivary glandular homogenate induced oviposition, yet synganglial homogenate did not. Although vaginal insertion induced both ovum maturation and oviposition, the effect was not as pronounced as when similar doses were administered by hemocoelic injection. These results indicate that a complex inter-related series of precopulatory and copulatory stimuli are necessary for oviposition to occur in fed O.

Relationships Between Weights of Nymphal Stages and Subsequent Production of Nymphs, Adults and Sex Ratios of O. parkeri

From a practical standpoint, with laboratory colonies it is often necessary to be able to predict how many adult ticks of a specific sex will be available for experimental use. Moreover, certain experimental designs

require treatment of ticks in the immature stages and scoring in subsequent nymphal or adult stages. In order to provide the kind of information needed the following experiments were conducted.

Unfed third stage nymphal (UN3) Ornithodoros parkeri were arranged into 7 weight classes, fed and reared to adults. Fed N3s produced 22.0% males, 3.8% females, and 57.2% fourth stage nymphs (N4s) (17.0% died or were lost). The resulting N4s were fed and subsequently produced 14.7% males, 56.4% females, and 9.5% fifth stage nymphs (N5s) (19.3% died or were lost). The N5s were fed and yielded 5.0% males, and 70.0% females (25.0% died or were lost). After rearing all UN3s to the adult stage the final sex ratio was 0.77:1 (43.5% males, 56.5% females). Unfed third stage nymphs weighing less than 2.6 mg produced males and N4s, but not females after feeding. A few females were produced from N3s heavier than 2.6 mg. N4s resulting from all N3 weight classes developed into males, females and N5s, and those originating from N3s in the lowest weight class (0.1-1.0 mg) produced more males than females. N4s originating from all other N3 weight classes produced more females.

In a second experiment, only females ecdysed from fed N4s weighing 25 mg and over, and those weighing 5-9 mg produced only males and N5s. Fifth stage nymphs in both experiments produced females almost exclusively (34 females and 1 male).

Spermatogenesis and Spermiogenesis

The processes and developmental timing of spermatogenesis and spermiogenesis in tick and parasitic mites continues to be investigated as well as the stimuli initiating these events. Karyotypes, sex chromosome systems and sex ratios all relate to background information needed when considering genetic linkage groups and various parameters of reproduction. During the

past 12 months we were able to make significant contributions to the data base on tick cytogenetics and developmental timing of sperm production. Four species of the medically important Amblyomma were studied. Amblyomma inornatum attached to the host for 1 to 3 days contain enlarging primary spermatocytes, but no meiotic divisions are evident.

Prophase I (especially diplotene and diakinesis), metaphase I, anaphase I and metaphase II are first seen on day 4 of attachment. These and subsequent meiotic stages as well as rounded spermatids are present on days 5 and 6 of feeding. Each subsequent day more cells develop into spermatids and fewer cells are seen in the meiotic divisional stages.

Cells at diakinesis and metaphase I reveal 10 autosomal bivalents plus one sex univalent. Reductional division occurs at anaphase I with the sex univalent going undivided to one pole along with 10 autosomal dyads, and 10 autosomal dyads without a sex chromosome go to the other pole. Anaphase II is equational. These 2 meiotic divisions result in the subsequent formation of 4 spermatids (2 with 10 + X and 2 with 10 chromosomes) from each primary spermatocyte.

Eleven pairs of chromosomes (bivalents) including 1 long pair are present in prophase I of oocytes. Later meiotic divisional stages of oogenesis are difficult to observe. Mitotic cells reveal 22 and 21 chromosomes from females and males, respectively. These include 20 autosomes (16 moderately long, 4 slightly shorter) in both sexes plus 2 long sex chromosomes (XX) in females and 1 long sex chromosome in males (X). Thus the diploid chromosome number of A. inornatum is 22 for females and 21 for males, and the sex determining mechanism is XX:X0.

The reptile feeding species A. dissimile (larvae feed on mammals also) has chromosomal configurations indicating terminal centromeres on all chro-

mosomes. Males have 20 autosomes plus 1 sex chromosome and females have 20 autosomes plus 2 sex chromosomes. Spermatogenesis is slightly delayed in this reptile feeding tick when compared to other Amblyomma that feed on mammals. A. dissimile normally reproduces bisexually, although there is a tendency for parthenogenesis in some individual females. The genetics of parthenogenesis is not understood in most cases. Another interesting aspect of the feeding and reproductive biology of this species is the habit of adults attaching to hosts in clusters. Although not tested, it seems likely that the clusters might be the result of an aggregation pheromone produced by the ticks.

Males of a new species of Amblyomma tick from the Galapagos Islands possess a diploid chromosome number of 21. Meiotic divisions reveal 10 autosomal bivalents and 1 long sex univalent. Another Galapagos tick, Amblyomma darwini has a chromosome number of 20 consisting of 9 autosomal bivalents and 1 sex bivalent. The X chromosome is the longest of the complement and the length of the Y chromosome is uncertain. Anaphase I is reductional and anaphase II equational for both species.

Feeding as an adult is necessary prior to meiotic divisions and spermiogenesis in metastriata ticks. After feeding the subsequent factor(s) triggering the initiation of spermatogenesis is not known but our working hypothesis supported by preliminary data is that an ecdysteroid plays an early role in this process. We are currently analyzing several samples from ticks that fed for varying lengths of time in an effort to score changes in ecdysteroid titer.

IX. SUMMARY

This project demonstrated hormonal regulation of mating behavior and reproductive activity in both the hard ticks (Ixodidae), the soft ticks (Argasidae) and in mesostigmatid mites (Mesostigmata). We demonstrated that the steroid hormones ecdysone and 20-hydroxyecdysone exist in the 2 species of ticks used as exemplary material in our studies, i.e., Dermacentor variabilis and Hyalomma dromedarii. In addition, we demonstrated that exogenous hormone, administered in physiological doses, accelerated production of the sex pheromone 2,6-dichlorophenol, and that radiolabelled hormone accumulated in the pheromone glands. We monitored the cycles of production and decline of the hormone throughout the various phases of the tick's life cycle and correlated the timing of pheromone gland growth and development with the periods of ecdysteroid activity in the tick's body tissues. In more recent experiments, we demonstrated the sensitivity of the sex pheromone gland system to excitation by Triol analogues of the natural hormone, especially 22, 25- dideoxyecdysone. We also demonstrated excitation of 2,6-dichlorophenol production by the sex pheromone glands maintained in vitro as well as in vivo, and showed that the addition of dopamine or the maintenance of the synganglion-gland neural connections enhanced synthesis. These and other related findings were reported in a series of papers published in refereed journals, proceedings of various international congresses and as chapters in forthcoming texts. We also called attention to the reported excitation of sex pheromone activity by 20-hydroxyecdysone in the housefly, Musca domestica, cited by Blomquist and his colleagues in several important publications. Clearly, there is evidence available from several different

sources that ecdysteroids play an important regulatory role in the adult arthropod. In view of this discovery, we focused our attention on ecdysteroids in ticks. The synganglion was implicated as a possible source of ecdysteroids in the mature virgin female, while more recent studies described in a forthcoming publication indicate an important role for the diffuse fat body. The metabolism of ecdysteroids was also described, including inactivation by conjugation with long chain polyunsaturated fatty acids. Following synthesis from cholesterol and, possibly, other sterol precursors, ecdysone is oxydized to 20-hydroxyecdysone, the primary hormone, small amounts of 20,26-dihydroxyecdysone and may be altered further to unknown deoxy derivatives. In contrast with its metabolism in the insects, no evidence of ecdysonic acids was observed.

We also explored the possible role of juvenile hormone in sex pheromone activity and in regulation of reproduction. No evidence of juvenile hormone (JH) excitation or inhibition of sex pheromone activity was found. However, in studies conducted by J. H. Oliver, JH administration partially restored reproductive capacity in chicken mites, Dermanyssus gallinae, in which it has been disrupted by treatment with Precocene-2. In the argasid tick, Ornithodoros parkeri, topical application of JH or injections of JH and Farnesol initiated vitellogenesis, but did not induce oviposition. Experiments with catecholamines indicate a role for these compounds in stimulating vitellogenesis. In experiments reported in a recent published paper, extracts of male accessory glands and salivary glands were found to initiate vitellogenesis and oviposition when inserted into the vulva of fed virgin females or inoculated into the haemocoel. Clearly, a complex interaction of chemical messages appears to be involved in the regulation of egg maturation and oviposition in this arthropod group.

In view of these findings implicating JH as part of a complex regulatory process involving several stimulatory molecules, we initiated studies to determine the presence of juvenoids in ticks. Preliminary assays done by Baehr using a highly specific radioimmunoassay revealed picogram quantities of a molecule similar or identical to JH-3, but not other juvenoids. Unfortunately, attempts to confirm this identification by GC/MS were unsuccessful. Study of the metabolites produced by incubation of authentic, highly purified labelled JH revealed 3 compounds, indicating the presence of a JH specific epoxide hydrolase. Photoaffinity affinity assays revealed the presence of JH specific binding proteins. All of these findings were made with hemolymph from part fed virgin females, the same physiological state found by Oliver to be responsive to artificial administration of this juvenoid. We believe that these findings provide important new evidence of the presence of juvenoids in ticks and their role in initiative reproductive activity. These studies are continuing and it is hoped that sufficient material can be accumulated to allow definitive chemical identification of this elusive hormone.

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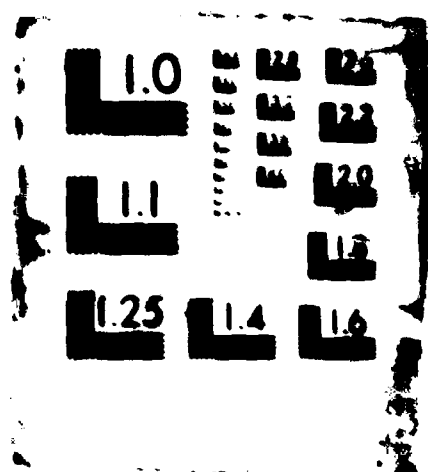
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